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HISTOCHEMICAL STUDIES ON VITAL AND POST-MORTEM SKIN WOUNDS

EXPERIMENTAL INVESTIGATION ON MEDICOLEGALLY SIGNIFICANT VITAL REACTIONS IN AN EARLY PHASE OF WOUND HEALING

BY

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J. R.

CONTENTS

I. INTRODUCTION

1. MEDICOLEGALLY SIGNIFICANT VITAL REACTIONS

Vital reactions in the medicolegal sense include all reactions of tissues and organs for which activities of living cells are necessary (Strassmann 1954). Ever since forensic medicine became an independent branch of science, the distinction between vital and post-mortem injuries has been one of its cardinal problems. As long ago as 1786 Plenk in his *Elementa Medicinae et Chirurgiae Forensis* emphasized the importance of recognizing vital reactions and differentiating them from post-mortem phenomena. A body may be accidentally damaged after death, and if such injuries are assumed to be the result of violence before death, an unwarranted suspicion of murder may be entertained.

In earlier textbooks, such as that by Hofmann and Haberda (1919), it was mainly the macroscopic or systemic vital reactions that were discussed. These consisted of haemorrhages, thrombi, emboli, aspiration and swallowing of blood and foreign bodies. Some observations were also made on local vital reactions visible to the naked eye in a wound. The extravasated blood was imbibed by the damaged tissue, the wound edges gaped and became swollen. Coagulated blood was found in and around the wound. Later on, signs of inflammation and of healing dominated the picture.

Walcher (1930, 1936) pointed out the importance of studying the lesions microscopically in order to distinguish between antemortem and post-mortem injuries. He noted that necrosis and inflammation were the main components of microscopic vital reactions. The signs indicating the vital origin of an injury became clearer as time elapsed after wounding. Orsós (1935) emphasized the difference between somatic and cellular death, the former

being initially characterized by cessation of respiration, heart contractions and circulation, and by loss of sensibility and reflexes. With somatic death not all cells and organs cease to function immediately; certain activities continue, and reactions of cells and tissues to stimuli can be produced by artificial means for a considerable time afterwards. Orsós, therefore, used the following classification:

1) intravital reactions, occurring when the whole organism is still able to function,
2) agonal reactions, seen just before somatic death,
3) signs of somatic death,
4) intermediate reactions, produced by individual cells still able to function after somatic death,
5) post-mortem reactions, seen after cellular death, and
6) signs of decomposition.

He made an attempt to distinguish between ante-mortem and post-mortem local phenomena by using the histological staining method of Mallory. Orsós used the term »metachromasia» to describe the difference in colour seen in connective tissue fibres injured *intra vitam* as compared with those in undamaged parts of the same preparation. He believed that this »metachromasia» was due to dehydration and to the relative increase in the globulin content of the proteins. Blum (1937) noted such a colour change, however, not only in wounds made during the intermediary phase but also in those cut after the dissolution of rigor mortis.

Cellular infiltration, indicating inflammation in a vital wound, was studied histologically by Walcher (1930). According to him a few leukocytes were seen three to five hours after wounding. Some other investigators reported leukocytic infiltration in a 12-hour wound (Smith 1945), or 13 to 18 hours after injury (Russell 1951). According to Masshoff (1957), the histological diagnosis of leukocytic infiltration and of the mobilization of histiocytes was one of the best criteria for distinguishing between vital and post-mortem injuries. There was, however, the disadvantage that no distinct leukocytic zone was visible until too much time had elapsed.

Ökrös (1938) claimed that retraction and aggregation of elastic fibres characteristic of vital injuries did not occur in post-mortem

wounds. Similar changes, however, were demonstrated in post-mortem lesions, too (Stössel 1950).

Using Weigert's stain for fibrin, Kernbach (1937) studied connective tissue fibres in vital haematomata. He considered that the appearance of blackish threads, consisting of both elastic and collagen fibres, indicated the vital origin of haematoma. However, in reinvestigations (Bahlmann 1939, Hilt 1950) similar formations were shown to be present in post-mortem haematomata, too.

Blum (1937) made a microscopical study of the strangulation mark and noted an emulsification in the fat cells constricted by the ligature if the hanging had taken place before death. In bodies hanged post mortem to conceal a homicide, no emulsification was seen in these cells. These results could not be confirmed later (Hubalek 1951).

The occurrence of haemosiderin in the neighbourhood of vital injuries was ascribed by Walcher (1930) to the phagocytosis of erythrocytes. He could not find haemosiderin until the ninth day after wounding. In an experimental study (Muir and Niven 1935) an iron reaction of a diffuse character was detected in cells in the vicinity of the wound within 24 hours. Granular haemosiderin appeared later and formation of iron-free haematoidin took place about the seventh day. Hallermann and Illchmann-Christ (1943) reported the appearance of haemosiderin on the third day in the vicinity of a vital injury. Neudert (1953), on the other hand, pointed out the possibility of a post-mortem resorption of blood.

Uotila (1943) used buffered solutions of methylene blue in order to determine indirectly the isoelectric point of fixed tissue. He detected changes in colour in vital wounds but not in post-mortem ones. The differences were, however, quantitative rather than qualitative, and the interpretation of the results required some experience.

In his famous textbook of forensic medicine, Mueller (1953) summarized the trustworthy vital reactions as follows: haemorrhages with abundant fibrin formation and resorption of blood by the neighbouring organs, signs of inflammation with phagocytosis, thrombosis, emboli, and aspiration of blood, etc. The shortness of the list is noteworthy. As an expression of total nihilism, Janezic-Jelacic (1956) denied the existence of dependable vital reactions.

Post-mortem phenomena and their possible effects on staining reactions should be taken into consideration when the true vital origin of an injury is to be determined by studying it microscopically. Lorke (1953) investigated post-mortem pH changes in tissues. He noted an abrupt drop to as low as pH 4.7 during the first 30 hours, followed by a rise up to pH 9.0 until the fifth day. Thereafter a slow decrease occurred within the alkaline range.

The post-mortem phenomena have been divided into two phases (Schmidt, Lorke and Forster 1959). The initial period of glycolysis is characterized by lactic acid production with a fall in pH. The phase of proteolysis begins about the second day. In aerobic conditions an oxidative breakdown of proteins takes place, producing carbon dioxide and ammonia. The pH increases sharply during this period. In anaerobic circumstances decomposition is slower, being reflected in a tardy increase in pH. More stable breakdown products, probably amines, are formed anaerobically.

Jetter (1959) investigated post-mortem biochemical changes in the blood, and likewise noted the decrease in pH secondary to lactic acid formation. The increase in plasma potassium and the decrease in the cell sodium content reflected increased permeability of the cell membranes.

The effect of post-mortem phenomena on certain histochemical reactions was studied by Kent (1957). Organs of dogs were incubated at + 37°C and samples were taken until 48 hours after death. The glycogen content decreased rapidly post mortem. The succinate dehydrogenase activity and the desoxyribonucleic acid content were markedly decreased 12 hours after death. The alkaline phosphatase activity was only slightly decreased after 42 hours. Considerable diffusion was noted, particularly after 12 hours' incubation. Smith *et al.* (1957) investigated changes in the levels of enzymatic activity by quantitative histochemical analyses after post-mortem intervals of two to six hours. Alkaline and acid phosphatases showed great stability. They concluded that significant results may be obtained by analyzing these enzymes in material collected within the intervals mentioned. Aminopeptidase activity, as studied microscopically also persisted for at least nine hours after death (Rosenholtz and Wattenberg 1961).

On the other hand, it is noteworthy that some enzymatic processes continue after somatic death. According to Strassmann

(1954), the proteolytic, glycolytic and lipolytic actions of enzymes lead to autodigestion of organs and to autolysis of cells. Other investigators (Gallagher, Judah and Rees 1956, Richterich 1958) have pointed out that the abrupt cessation of synthetic processes after somatic death, without a simultaneous interruption of the enzymatic breakdown of proteins, causes autolysis.

2. PHASES OF WOUND HEALING

The problem of wound healing has been approached from several points of view. Accordingly, there exist as many systems of classifying this process.

In addition to visual inspection, Carrel (1910) measured the successively decreasing surface area of skin wounds by planimetric methods. The wound remained the same size for two to five days, after which »granular retraction» and, finally, epithelization were seen. At his suggestion, a formula was devised (du Noüy 1916) by which it was possible to calculate the rate of healing of a skin defect.

Chlumsky (1899) described the principles of a method for determining the force required to tear healing wounds. He noted that the tensile strength decreased during the first four days and then increased up to the tenth day, when it was almost normal. On the basis of this observation and their own studies, Howes, Sooy and Harvey (1925) distinguished three phases of wound healing:

1) the lag or latent phase, with decreasing tensile strength, up to the fourth or sixth day;

2) the period of fibroplasia, lasting to the tenth or fourteenth day. During this phase the tensile strength rapidly increased, and fibroblasts were seen simultaneously;

3) the period of maturation, of varying length. At that time the scar assumed its definitive structure.

Histological studies on wound healing have been exhaustively reviewed by Arey (1936), Needham (1952), Gillman (1959) and others. In addition, Stearns (1940) and Harvey (1949) pointed out the formation of connective tissue fibres by fibroblasts. Other authors have emphasized the significance of the connective tissue

around hair follicles in the regeneration of skin (Bishop 1945), and of mitosis, especially in the repair of epidermis (Pinkus 1951, 1952). On the basis of their histological studies, Locallo, Casale and Hinton (1943) divided the lag period into a phase of traumatic inflammation and a phase of destruction.

Following Paget (1853) and Marchand (1901) and others, it is usual to distinguish between primary and secondary healing, according to whether the wound is closed or open. In open wounds the reactions of repair are qualitatively similar to those observed in primary healing. Quantitatively there are differences in regard to the duration of healing, the amount of granulation tissue etc. (Radvin and Zintd 1945).

By measuring the electric potential differences produced by rapidly dividing cells, Burr, Harvey and Taffel (1938) were able to distinguish two phases in wound healing. During the proliferative phase cell multiplication dominated up to the seventh day, after which the phase of cell differentiation began to have greater significance.

The earlier biochemical and histochemical investigations on wound healing have been reviewed by Arey (1936) and by Needham (1952). The latter divided wound healing into two phases: regression and repair. Even as recently as 1960, Cuthbertson has pointed out the existence of an »ebb» period of diminished vitality as the initial phase of regeneration, followed by a »flow» period of increased metabolism.

The prevailing view that metabolic inactivity characterized the earliest phase of wound healing was challenged by Dunphy and Udupa (1955). They reported the production of mucopolysaccharides already 24 hours after the injury, in amounts gradually increasing up to five days. In accordance with Abercrombie, Flint and James (1954), they observed the first chemical and histological evidence of collagen about the fifth or sixth day. Dunphy and Udupa therefore divided wound healing into two phases. The first they called the productive or substrate phase. During this period, from one to five days after wounding, mucopolysaccharides and soluble protein precursors of collagen were produced. The second or collagen phase then supervened, lasting until healing was complete. In this period normal collagen fibres appeared (Dunphy and Udupa 1955, Jackson 1958).

Even Dunphy and Udupa (1955), however, considered that in wound healing there occurs an initial, metabolically inert »shock» period of 12 to 24 hours' duration.

Thus, the length of the »lag» phase, as determined by physical and histological techniques, varied from four to seven days after wounding. Some biochemical and histochemical observations reduced it to approximately one day. Efforts to shorten the »lag» phase even more (Hershey and Mendle 1954) have been unsuccessful.

3. HISTOCHEMISTRY AS A MEANS FOR VISUALIZING EARLY FUNCTIONAL CHANGES

As Cameron and Hasan (1958) have pointed out, new methods must be developed that will be sensitive enough to detect early functional changes. For this purpose histochemical techniques seem to be the methods of choice, because they are able to give information not obtainable by other means (Wachstein 1955, Pearse and Macpherson 1958). Pearse (1958), in addition, emphasized that histochemistry is particularly concerned with the correlation of structure and function.

According to Bensley (1959), histochemistry is the study of the chemical and physical organization and reactions of the living elements and their products in the various tissues of the body. Microscopic histochemistry is based on the production of microscopically visible reactions when chemical and physical tests are applied to tissue sections prepared for microscopic study.

Histochemical methods, and in particular those for enzymes, have rel tively seldom been used in the study of wound healing. This is scarcely surprising in the light of the facts reviewed by Pearse (1958). Apart from a few historical methods which were in existence in the early years of this century, enzyme histochemistry can be considered to date from the end of the fourth decade. It was then that Gomori (1939) first described his method for alkaline phosphatase. By 1953, histochemical methods had been evolved for the demonstration of 18 different enzymes. In 1958, the figure stood at 45. Pearse (1958) found this number not very imposing when compared with the grand total of some 700 recorded enzymes. The distribution of the 45 methods among the main enzyme divi-

sions had gradually become, however, more even than it has been only a few years before.

On the other hand, the functional significance of many histochemical enzyme reactions is still obscure. In general, moreover, the information provided by histochemical staining techniques is only rough and qualitative, and not exact or quantitative (Wachstein 1955). Efforts to quantitate subjectively should, therefore, be considered critically.

According to Bensley (1959) the three main limitations of histochemistry are as follows:

1. The tests, in general, serve to identify certain classes of substances rather than the precise chemical composition of any substance.
2. The tests are, at present, far from standardized.
3. The interpretation of the results of microchemical tests applied to tissue sections is fraught with pitfalls.

Gomori (1950) subdivided these pitfalls, particularly in regard to enzyme histochemistry, into three groups:

1) false negative reactions, *i.e.* a failure to get a positive reaction in spite of the known presence of the enzyme in the tissue examined. This could be due to fixation in unsuitable media, too feeble activity in the tissue, or anything increasing the rate of diffusion and solubility of the end-product or decreasing its formation;

2) false positive reactions, *i.e.* a reaction misleadingly similar to the genuine one but not due to an enzyme contained in the tissue. For instance, blackish pigment, preformed calcifications in tissue (when the method depends on deposition of calcium phosphate at sites of enzyme activity), adsorption on certain tissue elements, and bacterial hydrolysis of the substrate might cause false positive reactions;

3) false localization, *i.e.* a positive reaction due to enzyme contained in the tissue itself but appearing at sites other than the true primary microscopic location. This pitfall is due to diffusion artifacts.

Thus, to avoid, as far as possible, the most usual sources of error mentioned histochemical staining methods should preferably

be used for demonstrating qualitative changes. In a biological process, such as wound healing histochemical reactions can visualize and localize the first appearance of a given change. To diminish false negative reactions an adequate mode of fixation and an optimal pH are or special importance. By the use of coupling azo dye techniques in place of earlier methods for some enzymes, false positive reactions have been reduced. False localization is often due either to supersaturation of the incubation medium with the products of hydrolysis or to excessive incubation times, and can be avoided accordingly.

The criticisms discussed must be kept in mind. On the other hand, Montagna (1956) has pointed out that all histological appearances are artifacts of one kind or another, *i.e.* they represent the consequences of optical, mechanical and chemical transformations of the living system. He further states that an artifact is only mischievous if one fails to inquire into the credentials of the transformation process or assumes that no such process has taken place.

II. PROBLEMS

Our knowledge of trustworthy local vital reactions is relatively scanty and somewhat contradictory, as pointed out in the introduction. Many of the reactions so far studied either appear post mortem as well, or, if solely intravital, occur too late to make possible the medicolegal distinction between vital and post-mortem wounds. This is in conformity with the common belief in a »lag» period of considerable length in the beginning of wound healing. The doctrine of the metabolic inactivity of this initial phase finds expression in its several names: »lag», »latent», »regression», »ebb», or »shock» period.

It is known that histochemical methods, and those for enzymes in particular, are able to reveal early functional changes not detectable by conventional histological techniques. It therefore seemed likely that histochemical methods could provide useful information on medicolegally significant vital reactions in the earliest phase of wound healing.

For this purpose it seemed necessary to answer the following questions:

1. At what time do certain histochemically demonstrable changes, in particular those due to enzymes, appear in an early phase of healing of experimental skin wounds?
2. Is it possible, by histochemical methods, to visualize these changes even for a considerable time after the animal's death?
3. Do false positive staining reactions occur post mortem, simulating the vital phenomena demonstrable by the methods used?

III. MATERIAL AND EXPERIMENTAL METHODS

1. CHOICE AND MANAGEMENT OF EXPERIMENTAL ANIMALS

Hair replacements in the rat (Butcher 1935), mouse (Chase and Montagna 1951, Chase, Montagna and Malone 1953, Chase, Rauch and Smith 1951, Argyris 1954), and rabbit (Whiteley and Chadially 1954) is discontinuous and occurs in cyclical waves of active growth. In the rat and mouse, waves of activity start on the ventral surface and spread dorsally over the trunk. All the hair follicles within the area of advancing growth are at approximately the same stage of development (Montagna 1956).

There are many differences between active and quiescent skin, *e.g.* hair follicles in the active zone show significantly more mitoses (Chadially 1958) and a higher cytochrome oxidase activity (Carruthers, Quevedo and Woernley 1959) than follicles in the quiescent areas. The whole skin is several times thicker when hair is growing than when it is resting (Montagna 1956). Furthermore, skin with growing hair contains three times as much glycogen per gram of tissue as skin with resting hair (Montagna 1956). Thus, the physiological status of the skin is in many ways tied up with that of the hair follicles. The degree of injury which can be inflicted upon mouse skin appears also to depend upon the stage of development of the existing hair follicles. Maximal damage occurs in resting areas (Chase and Montagna 1951).

By contrast, in the guinea-pig and in man hair growth is mosaic, *i.e.*, each follicle goes through a cycle independently of its neighbours (Dawson 1930, Montagna 1956). So hairs in anagen (active proliferation), catagen (cessation of growth) and telogen (resting stage) may be seen side by side in any small area of skin in the guinea-pig (Chadially 1958).

Thus, because of its mosaic hair growth the guinea-pig seemed to be the most suitable experimental animal for studies on skin wounds.

According to Billingham and Medawar (1948), guinea-pig's skin consists of three major layers:

1) epidermis,
2) a thick dermis, closely knit to
3) a layer of striped muscle, the »panniculus carnosus».

The principal blood vessels and lymphatics of the skin run between the dermis and the panniculus carnosus, but the guinea-pig lacks the fascial layer of connective tissue fibres seen in the rabbit at this level. The epidermis of the guinea-pig is composed of a living stratum Malpighii and a dead, horny, superficial stratum corneum. Well-defined basal, spinous and granular layers can be distinguished in the stratum Malpighii (Montagna 1956).

The environmental conditions of the experimental animals were kept constant in order to avoid, as far as possible, errors due to changes in the general factors that affect wound healing. Localio, Casale and Hinton (1943) reviewed these factors as follows: age, temperature, water balance, acidosis and alkalosis, starvation, plasma protein level, vitamins C and A, hormones, and manifestations of certain diseases. Cameron (1952) added the effect of ultraviolet light and the state of general circulation to this list, and Taubenhaus (1952) summarized the effects of various hormones upon healing.

With these factors in mind, 40 healthy male guinea-pigs, all of them six months old, of the same laboratory stock were used. The weight of the animals varied from 550 to 600 g. The temperature in the animal room was controlled at about + 18°C. The only light was natural day light; electric lights were seldom used, although the experiments were carried out in the winter. The guinea-pigs were fed once a day at the same hour and their standard diet consisted of oats, hay, and rutabaga; the last-mentioned feed also served as a source of water. During the experiments ten animals, at most, were kept in one cage, measuring 1 × 1 m. The guinea-pigs in each cage were treated as a group. When wounded, each animal was kept in a separate cage lest additional injuries should be caused by other guinea-pigs of the group.

2. PERFORMANCE OF EXPERIMENTS

Two circular wounds, 5 mm in diameter, were cut with sharp earmarkscissors in a dorsal area of the skin slightly elevated with the fingers. The area, located on the left side of the animal, was washed with soap and shaved immediately before the operation. No anaesthesia was used. The centres of the circles were 2 cm from the midline, the anterior one being situated immediately behind the shoulder blade. The posterior one was straight behind it, about 2 cm caudally of the anterior wound. The skin inside the circles was excised with fine scissors. Neither ligatures nor sutures were used.

Nine guinea-pigs were killed by decapitation without anaesthesia at intervals of 1/2, 1, 2, 4, 8, 16, 32, 64, and 128 hours, respectively, after wounding. Flaps of skin containing the circular wound were removed immediately. The anterior flap was used for histochemical demonstration of enzymes. One half of it was frozen fresh with solid carbon dioxide, and the other half fixed for 10 hours in cold (+ 4°C) neutral buffered 10 per cent formalin (Lillie 1954). The posterior flap was used for the demonstration of other substances and for histological studies. It was fixed for 24 hours at room temperature in neutral buffered 10 per cent formalin.

For the study of any false positive staining reactions possibly occurring after death, similar wounds were made at the same post-mortem intervals of 1/2, 1, 2, 4, 8, 16, 32, 64 and 128 hours, respectively, on the right side of the corresponding guinea-pig, kept at about + 18°C. Thus, for instance, on the back of the animal wounded two hours before death, the post-mortem injuries were made two hours after death. Flaps of skin surrounding the post-mortem wounds were removed as soon as the excisions had been made. Here, too, the anterior flap was used for the demonstration of enzymes and the posterior one for the other tests. Fixations were made accordingly (see Fig. 1).

In order to investigate the post-mortem preservation of vital reactions appearing in the healing wound, the tenth guinea-pig was killed 48 hours after wounding. These wounds, five of them on each side of the back, were made as described previously. The most anterior couple of skin flaps was removed 24 hours after

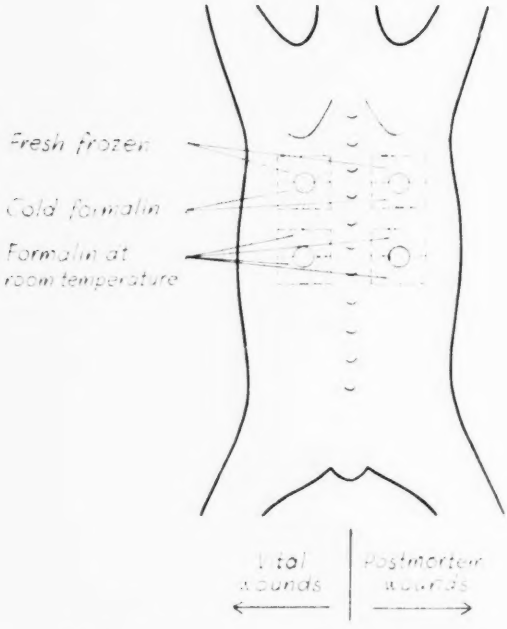

Fig. 1. — Schematic diagram showing the positions of the wounds and the types of fixation used.

death, the second after 48 hours, the third three days, the fourth four days, and the fifth five days after death. The left flap was used for enzyme studies, and the right one for the demonstration of other substances. The system of fixations was analogous to that described previously.

In order to avoid the possible error produced by individual differences, four replicate series of experiments were made, each consisting of 10 guinea-pigs.

3. HISTOCHEMICALLY DEMONSTRABLE CHANGES STUDIED

As a working hypothesis it was assumed that hydrolases might begin to act soon after wounding, causing breakdown of some cell constituents. Accordingly, the activities of non-specific acid and alkaline phosphatases and aminopeptidase, as representatives of this group of enzymes, were studied histochemically. Since injury might modify the respiratory processes of the cells, it seemed

worth trying to study the activities of cytochrome oxidase and succinate dehydrogenase, as representatives of oxidative enzymes.

Further, it was assumed that nucleic acids might be essential in the synthetic processes of healing, and according to Dunphy and Udupa (1955), an accumulation of acid mucopolysaccharides indicates the earliest phase of collagen formation. Hence it was also deemed useful to study nucleic acids and acid mucopolysaccharides.

In the following, the histochemical methods used are discussed and described in detail in connection with the particular changes or substances which they are used to demonstrate.

Attention is paid to the epidermis and the dermis, since these are homologous with the corresponding parts of human skin.

1. EVALUATION OF RESULTS

It is known that the intensity of the histochemical staining reaction rarely bears a simple, stoichiometric relation to the concentration of the reactive material in the section (Eränkö 1955). Therefore, an attempt at an exact quantitation of the results did not seem warranted. Further, the purpose of this study was not to obtain quantitative results but to collect qualitative data concerning the appearance of certain histochemically demonstrable changes in wounds.

In order to make the characterization of the results clearer, however, the intensity of staining was indicated by plus symbols (1 plus, 2 plus and 3 plus). This type of »quantitation» is, of course, subjective and has a descriptive value only. It may, however, serve to indicate some changes from one magnitude class to another. The reactions used for the demonstration of enzymes, on the other hand, represent changes in activity which do not inevitably involve changes in amount. Nevertheless, these very reactions may reveal alterations of great functional significance, as pointed out in section I, 3. of the present publication.

Because of possible species differences, the results obtained with animal experiments are not directly applicable to man. Most of the usual laboratory animals have a more rapid metabolism than man. This might cause faster reactions in wound healing.

Extensive studies on autopsy cases are, therefore, necessary before the results of this study can be applied to medicolegal practice. Nevertheless, in the following discussions of each phenomenon studied, the medicolegal point of view will also be taken into account. For it is believed that even the results of animal experiments may to some extent reflect the sequence of events occurring in wound healing in general.

IV. BRIEF SURVEY ON HISTOLOGICAL CHANGES

As a standard of comparison a brief survey of the changes in wounds demonstrable by a conventional histological technique will be given before the sections dealing with the histochemical studies proper.

1. EARLIER INVESTIGATIONS

The extensive literature relating to histological studies on wound healing has been reviewed by Arey (1936), Needham (1952), Allgöwer (1956), Gil'man (1959), and Johnson and McMinn (1960). Ehrich (1956) was especially concerned with traumatic inflammation in his exhaustive survey, the bibliography of which consisted of about 2 400 references. A number of other authorities, *e.g.* Whipple (1940), Harvey (1949), Cuthbertson (1960), Trémolières and Derache (1960), as well as many standard textbooks, *e.g.* those by Christopher (1945), Berman (1950) and Hellner, Nissen and Vosschulte (1958), are unanimous in accepting the occurrence of a metabolically inert »lag» phase of one to five days' duration at the onset of wound healing. This opinion is based on the results of physical and histological studies. Firket (1951 a) investigated the healing of guinea-pig skin wounds, and observed an inflammatory reaction during the first two days. The earliest signs of regeneration appeared on the third day. According to Pepper (1954), the formation of granulation tissue, and the inward migration of epithelium from the wound edges began in this animal species between three and five days postoperatively.

2. HISTOLOGICAL METHOD

For histological study halves of the posterior skin flaps (see Experiments) were fixed for 24 hours at room temperature in

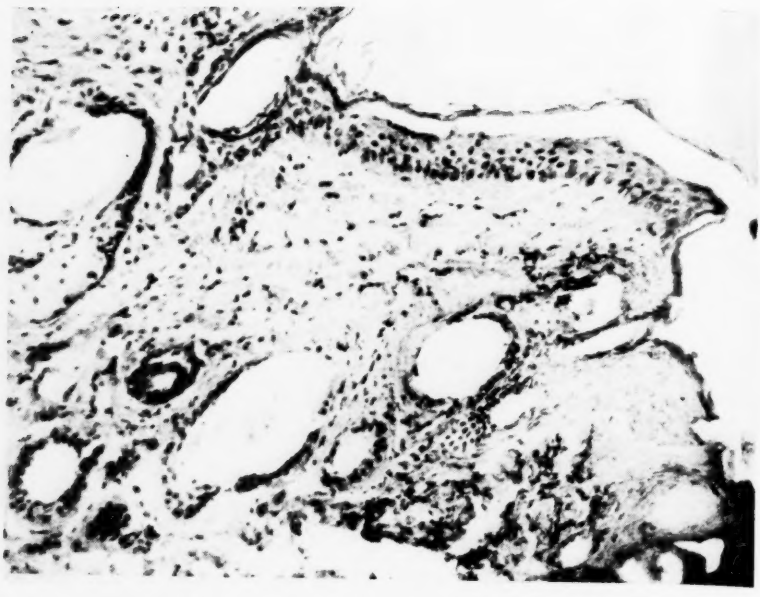

Fig. 2. — Histological view of an 8-hour vital wound. The accumulation of leukocytes is beginning. (Weigert — van Gieson, × 100)

neutral buffered 10 per cent formalin. The specimens were dehydrated, cleared and mounted in paraffin in the usual way. Sections were cut at 10 μ and stained by the van Gieson picrofuchsin variant of Weigert's acid iron chloride haematoxylin method (Lillie 1954).

3. RESULTS

In the first two hours after the vital injury, bleeding and exsudation dominated. The tissues became swollen owing to capillary and arteriolar dilatation. In a 4-hour vital wound some polymorphonuclear leukocytes had migrated out of the vessels.

In the vicinity of an 8-hour excision, polymorphonuclear leukocytes were more numerous (Fig. 2), but they did not constitute a well defined zone around the lesion. In the innermost part of the wound the cytoplasm of the connective tissue cells had become swollen, and karyolysis or karyorrhexis was beginning in their nuclei.

In 16-hour vital wounds (Fig. 3) it was possible to distinguish a central zone 200 to 500 μ in depth from a peripheral wound zone

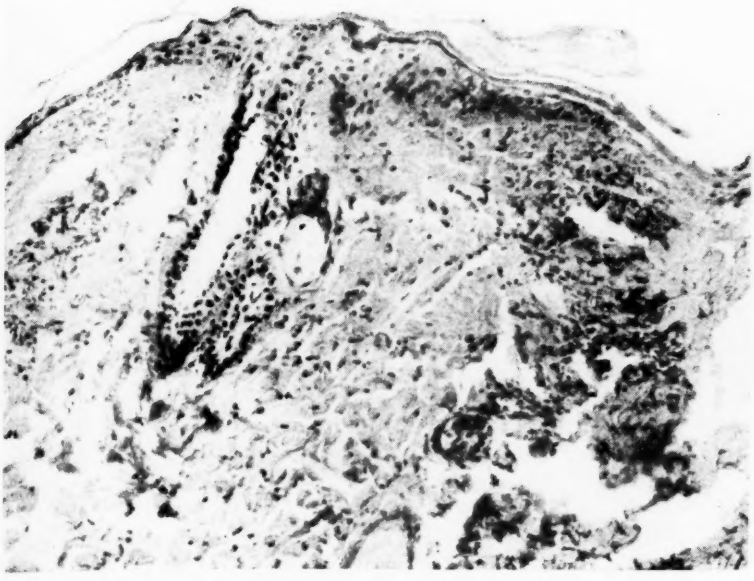

Fig. 3. — Histological view of a 16-hour vital wound showing the peripheral zone with migrated cells, and the advancing necrosis in the central area (on the right in the picture). (Weigert — van Gieson, × 100)

100 to 300 μ deep. In the latter mononuclear cells were seen, some of them, with evident phagocytic properties, containing cellular debris. The polymorphonuclear leukocytes were beginning to degenerate. In the central zone karyolysis and karyorrhexis had progressed in the connective tissue cells, the epidermal cells maintaining their staining properties better.

At 32 hours after the vital injury, round cells with large, spherical nuclei were principally responsible for the increase in the number of cells in the dermis of the peripheral zone. The increase in cell size, on the other hand, was chiefly due to the enlarged nuclei seen in that area. The nuclei of the endothelial cells of the capillaries also appeared swollen. The perivascular area was infiltrated mainly by mononuclear cells. Only occasional mitotic figures were seen within the connective tissue. The epithelial cells of the peripheral zone appeared to have enlarged. A few isolated mitoses were seen in the stratum basale. In the central zone necrosis had become more apparent in the connective tissue, the nuclei being hardly stainable.

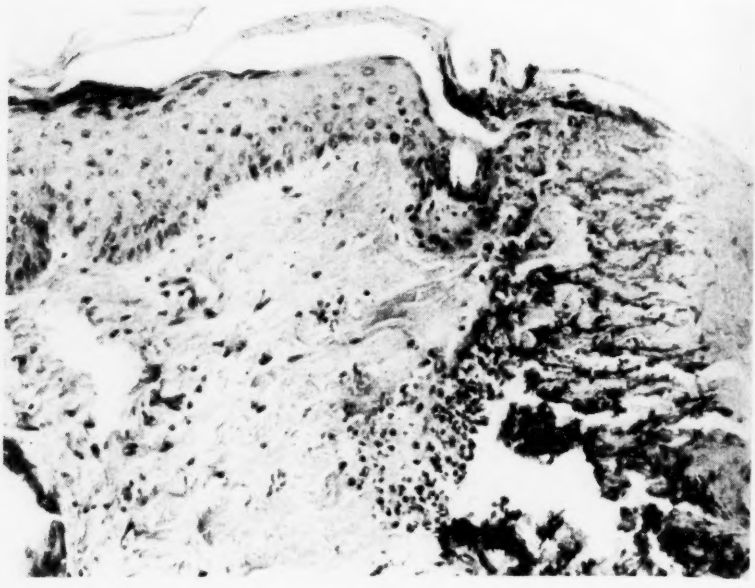

Fig. 4. — Histological view of a 64-hour vital wound. The accumulation of cells in the peripheral zone and the necrosis in the central one (on the right in the picture) are still more pronounced. (Weigert — van Gieson, × 100)

In the dermis of 64-hour vital wounds (Fig. 4) the round cells with their large, spherical nuclei, and the more elongated cells with oval or flattened nuclei together constituted an easily distinguished peripheral zone. Some mitoses were also seen in the connective tissue, but mitotic figures were more numerous in the lower layers of the thickened epidermis of the peripheral zone (Fig. 5). The central zone showed advanced necrosis in the connective tissue, which appeared as a nearly homogenous mass without stainable nuclei. The cell boundaries and the nuclei were better preserved in the epidermis and in the hair follicles.

At 128 hours after the vital injury the picture was in many respects similar to that seen 64 hours earlier. In addition, endothelial cells gave rise to new capillary loops around which tissue building occurred as granulations. The migration of epithelial cells was beginning at the wound edge.

The vital phenomena described were recognizable up to five days after death. No such distinct changes were observed in the wounds made post mortem.

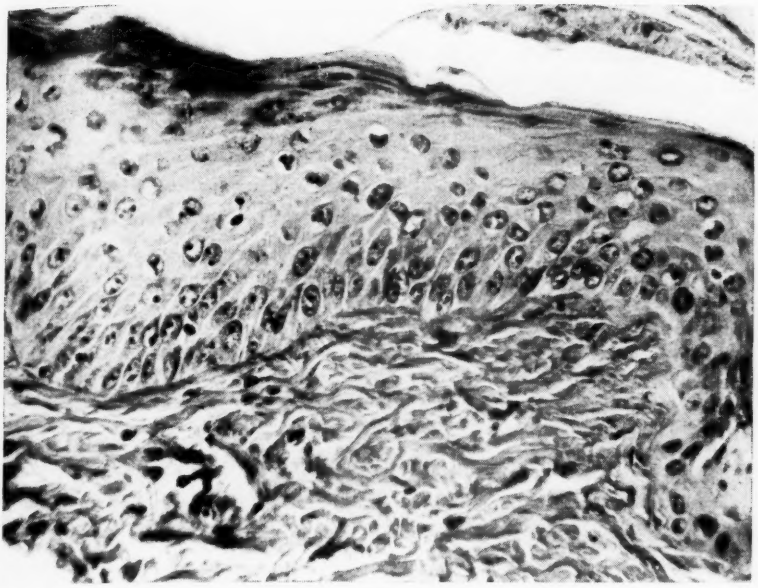

Fig. 5. — Histological illustration of mitotic figures in the lower layers of the thickened epidermis in the vicinity of a 64-hour vital wound (Weigert — van Gieson, × 400)

4. DISCUSSION

The results of this study are in conformity with those of a number of other workers, *e.g.* Borrel (1893), Marchand (1901), Menkin (1934), Hudack and Blunt (1950), Rebuck (1952) and Allgöwer (1956), who have pointed out that the initial migrating cells of an inflammatory reaction or of wound healing are polymorphonuclear leukocytes, followed later by mononuclear cells. On the basis of his earlier studies, Menkin (1955) attributed this cytological sequence to the inability of polymorphonuclear leukocytes to survive when the hydrogen ion concentration became high owing to lactic acid formation in the wound tissue. Mononuclear phagocytes appeared to be unaffected by the local acidosis developing.

It was very difficult to distinguish between the different species of mononuclear cells appearing in the outer zone from 16 hours after the vital injury. Some of these cells were elongated with oval or flattened nuclei, resembling the connective tissue cells

of the uninjured dermis. In the following description of the histochemical changes, cells showing these morphological characteristics will be called fibroblasts. The other main variety, with large, spherical nuclei in a round, relatively ample cytoplasm, will be called mononuclear phagocytes, since many of these cells showed phagocytic properties. Intermediate forms were observed, however, and the two contrasted types are probably merely functional or developmental stages of the same cell.

The results of the histological studies were in keeping with a great number of earlier investigations (Arey 1936, Firket 1951 a, Teir, Putkonen and Kiljunen 1951, Teir, Kiljunen and Putkonen 1951, Pepper 1954, Allgöwer 1956, Ehrich 1956, Gillman 1959). The first trustworthy signs of regeneration, several mitoses, were observed in a 64-hour vital wound. According to Teir (1952), the latent period preceding an increase of mitotic activity is necessary for the formation of new cytoplasmic and nuclear components which, having reached a certain chemical »ripeness», release mitotic cell division.

The cellularity of the outer dermal zone greatly increased, however, even after 16 hours following the vital injury. The origin of the new mononuclear cells in the healing connective tissue is still a matter of debate. Some authorities assume that these cells are derived from pre-existing fibroblasts (Virchow 1871, Ham 1950, Edwards and Dunphy 1958), others regard them as migrating from the blood (Maximow 1902, Allgöwer 1956, McMinn 1960), or from both the tissues and the blood (Boyd 1961). Since mononuclear cells are seen in the outer zone before a significant increase in mitotic activity occurs, it seems apparent that the major source of these cells is not a local one.

Assuming that migrated round cells participate (Allgöwer 1956) in the formation of new connective tissue, *i.e.* in a repair phenomenon, the »lag» period could be shortened to at most 16 hours after the injury. It seems, accordingly, that the length of the »lag» phase depends on the particular aspect of healing studied.

From the medicolegal point of view the appearance of a peripheral zone, consisting of mononuclear cells in the dermis, after 16 hours, and of proliferating epidermis after 64 hours, was a histologically demonstrable vital change like the accumulation of polymorphonuclear leukocytes from eight hours on. The increase

in cellularity may be called a positive vital reaction in contrast to the negative one occurring in the central zone, where necrosis became apparent after the 16th postoperative hour. The progressive necrosis, with its typical nuclear changes, could easily be distinguished from post-mortem autolysis, in which the nuclei preserved their stainability during the experimental period. Neither migration nor mitosis occurred in the vicinity of the wounds made after death. According to these experiments, the conventional histological technique may thus be used to distinguish between vital and post-mortem wounds, provided that the victim survives a considerable time after the injury, *i.e.* at least eight hours, when the cellularity increases distinguishably.

5. SUMMARY

Traumatic exsudation was followed by infiltration of polymorphonuclear leukocytes, which were distinctly detectable in 8-hour vital wounds. They were replaced by mononuclear cells which constituted a definite peripheral wound zone from 16 hours after the injury (positive vital reactions). Progressive necrosis in the central zone from the same point of time was regarded as a negative vital reaction. Numerous mitoses, another positive vital phenomenon, were not seen until 64 hours after the injury. Since no such reactions were seen in the wounds made after death, and since the vital changes were easily recognizable up to the fifth post-mortem day, the conventional histological method can be used to distinguish 8- to 16-hour or older vital wounds from post-mortem ones.

V. STUDIES ON ALKALINE PHOSPHATASE ACTIVITY

1. EARLIER INVESTIGATIONS

Phosphatases are enzymes able to hydrolyze esters of phosphoric acid. According to Wachstein (1955) the phosphatases may be classified into three groups on the basis of substrate specificity and pH optima:

1) non-specific alkaline phosphatases;
2) non-specific acid phosphatases;
3) specific phosphatases (*e.g.* 5-nucleotidase).

Non-specific alkaline phosphatases hydrolyze many phosphomonoesters to phosphoric acid and the alcohol radical, particularly at pH 9.0 and above.

The localization of alkaline phosphatase activity in guinea-pig skin has been studied by Danielli, Fell and Kodicek (1945). They have demonstrated that the sebaceous glands, the proximal part of the hair follicles, and a few capillaries give a strong reaction for this enzyme, but the rest of the skin and the subcutaneous tissue do not stain. Alkaline phosphatase activity has also been observed in the stratum granulosum of human and guinea-pig epidermis by some authors (Fisher and Glick 1947, Smith and Parkhurst 1949, Lansing and Opdyke 1950); while others have detected only a weak staining in this layer (Pirilä and Eränkö 1950, Montagna 1956). Firket (1951 b) and Kopf (1957) have stated that no true alkaline phosphatase activity is demonstrable in any epidermal strata. According to Spier and Martin (1956), too, alkaline phosphatase activity in the epidermis is an artifact, but a distinct reaction for the enzyme occurs in some parts of the cutaneous appendages.

Alkaline phosphatase activity has been shown in human and guinea-pig neutrophilic leukocytes, but not in lymphocytes, monocytes, or eosinophils (Wachstein 1946, Valentine and Beck 1951, Wachstein 1955). The number of stained neutrophils clearly increases in infections (Wachstein 1946). Elevations in unit leukocyte alkaline phosphatase have been noted in a wide variety of »stress» conditions: diseases, infection, trauma, pregnancy, and operative procedures (Valentine *et al.* 1954). The artificial nature of the nuclear staining, demonstrated by the calcium phosphate method in neutrophilic leukocytes, has been emphasized (Wachstein 1955, Kaplow 1955, Monis and Rutenburg 1959).

Several theories exist concerning the possible functions of alkaline phosphatase *in vivo*. This enzyme has been related to the calcification process (Bourne 1956, Henrichsen 1956), directly or indirectly to the intermediary metabolism of the nucleproteins, neutral fats, and glycogen (Atkinson and Engle 1947), to glycogenesis (Wislocki and Dempsey 1945, Johnson, Butcher and Bevelander 1945, Johnson and Bevelander 1946, Martin 1949) or to active solute transfer across cell boundaries (Emmel 1946, Dempsey and Singer 1946, Pritchard 1947, Bradfield 1950, Eränkö and Niemi 1954). In the last mentioned connection it is interesting that alkaline phosphatase activity has been demonstrated in the secretory epithelium of sweat glands (Bunting, Wislocki and Dempsey 1948), and in the walls of the capillaries (Paterson, Mills and Moffat 1957, Monis and Rutenburg 1960).

Fell and Danielli (1943) investigated the distribution of alkaline phosphatase in experimental skin wounds in rats. During the first days after the injury only the invading leukocytes gave an intense staining reaction. On about the fifth day there was a spectacular increase in reactivity, especially in the regenerating collagen. They concluded that this enzyme was concerned in the laying down of collagen. Even fibroblasts and capillaries stained deeply. In 3-day wounds in guinea-pig skin, Danielli, Fell and Kodicek (1945) found that alkaline phosphatase activity was strong in the leukocytes and in the scab, and weaker in the fibroblastic nuclei. On the sixth day, in addition, the capillaries and the dermal outgrowth immediately beneath the growing edge of the epidermis gave a strong reaction.

The theory that alkaline phosphatase participates in the forma-

tion of fibrous protein in wound healing (Fell and Danielli 1943, Danielli 1946, Bradfield 1946, Jeener 1947) has been challenged (Robertson, Dunihue and Novikoff 1950). According to some investigators (Gold and Gould 1951, Gould and Gold 1951) the strong affinity of collagen for phosphatase suggests that the enzyme present on the fibres in healing wounds may have been adsorbed from the surrounding tissue fluids. Balazs and Holmgren (1950) demonstrated abundant cells rich in alkaline phosphatase in the tissue closest to the edge of rat skin wounds only 24 hours after operation. Strong activity of this enzyme has been shown (Junqueira 1950) in the proliferating fibroblasts of tadpole tails, beginning approximately on the fourth day of regeneration. Firket (1951 b) reported alkaline phosphatase in the leukocytes only during the first days of healing. On the fourth day the mitotic nuclei exhibited strong enzyme activity. French and Benditt (1954) claimed that the regeneration of epithelium might determine phosphatase activity rather than fibre formation. Dunphy (1959) related the activity of this enzyme in the wound to the general phenomenon of cell proliferation.

2. HISTOCHEMICAL METHOD

Histochemical methods for revealing phosphomonoesterase activity are based on the demonstration of one or the other of the reaction products: phosphoric acid and the alcohol radical.

Gomori (1939) and simultaneously Takamatsu (1939) described a method depending on the precipitation of the released phosphoric acid as calcium phosphate, which was subsequently visualized. This method was criticized even by Gomori himself (1951), and the difficulties of interpretation of the results are discussed in section I, 3. of the present publication.

Menten, Junge and Green (1944) devised the first method for the histochemical demonstration of alkaline phosphatase activity depending on the alcoholic part of the phosphomonoester used as substrate. The new simultaneous coupling principle was based on the hydrolysis of calcium β-naphthyl phosphate and the instant reaction *in situ* of the liberated β-naphthol with diazotized α-napthylamine at pH 9.0 to give a red precipitate at the sites of phosphatase activity. The necessity for the preparation of a fresh

diazonium salt for every bath was overcome by forming a stable diazotate of α-naphthylamine (Manheimer and Seligman 1949), and the readily soluble sodium α-naphthyl phosphate was substituted for the relatively insoluble calcium salt (Friedman and Seligman 1950). Since the acetone fixation and paraffin embedding used by Gomori (1951) destroyed at least 70 per cent of the alkaline phosphatase present in the tissue, a short period of fixation in cold formalin and the use of frozen sections (Grogg and Pearse 1952 a) seemed preferable.

On the grounds of these facts the coupling azo dye method evolved by Grogg and Pearse (1952 a) was chosen for the demonstration of alkaline phosphatase activity in this study. The following technique was used:

1. The halves of skin flaps were fixed in 10 per cent neutral buffered formalin at + 4°C for 10 hours.
2. The specimens were rapidly frozen with solid carbon dioxide.
3. Sections were cut at 15 μ with a rotary microtome in a cryostat at — 20°C.
4. The sections were mounted on clean slides without adhesive and allowed to dry at + 20°C for three hours to ensure adherence.
5. 20 mg of sodium α-naphthyl phosphate was dissolved in 20 ml of 0.2 M-stock »Tris» buffer (pH 10.0) and 20 mg of Fast violet B was added, the mixture being stirred well. (The nomenclature of the stable diazonium salts used in this work, all of them produced by I.C.I., Ltd., follows the system proposed by Lillie (1959). Chemically, Fast violet B is the diazonium salt of 4′-amino-6′-methyl-*m*-benzanisidine). The mixture was filtered onto the sections, which were then incubated at + 20°C for 15 minutes.
6. The preparations were washed in distilled water for three minutes.
7. The sections were mounted in glycerin jelly.

Control sections were incubated without the substrate.

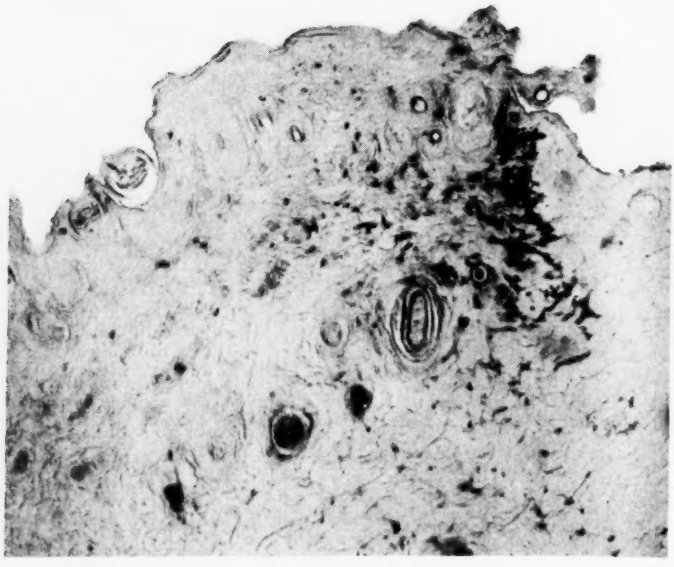

Fig. 6. — Alkaline phosphatase activity in a 4-hour vital wound. (Coupling azo dye method of Grogg and Pearse, × 100)

3. RESULTS

The sites of alkaline phosphatase activity stained dark brown. Control sections were colourless. No significant differences in the appearance of the active zone could be detected between the four replicate series of experiments each made with 10 animals. In the following description of the results, therefore, attention will only be paid to the time and staining variables. This applies to all the changes studied histochemically in this work.

In the uninjured guinea-pig skin none of the epidermal strata nor the hairs showed any demonstrable alkaline phosphatase activity (Fig. 6, to the left of the picture). A distinct (1 plus) reaction was observed in the papilla and in the proximal part of the external connective tissue sheath of the growing hair follicles. The capillaries and some fibroblasts of the dermis likewise took up the stain (1 plus).

In the injured skin the epidermis also remained unstained. No distinct changes were seen in the dermis until four hours after injury.

Fig. 7. — Alkaline phosphatase activity in an 8-hour vital wound. The central wound zone is on the right in the picture. (Coupling azo dye method of Grogg and Pearse. × 100)

For rough orientation the wound edge with its surroundings (on the right in the picture, cf. Fig. 6) was called the central zone, its depth being of the order of 200 μ to 500 μ. Beyond this a 100 μ to 300 μ deep surrounding area was referred to as the peripheral zone. The depth of the two zones varied within the limits mentioned irrespective of the time elapsed since wounding. No consistent differences in the depths in question were observed between the four replicate series of experiments.

In a 4-hour vital wound (Fig. 6), the intensity of staining began to decrease a little in the central zone as compared with the value (1 plus) observed at the corresponding sites of the uninjured skin. Simultaneously, in the dermis of the peripheral zone, a distinct (1 plus) staining was observed in a greater number of fibroblasts. In some of these cells the enzyme activity increased (2 plus). Solitary polymorphonuclear leukocytes showing alkaline

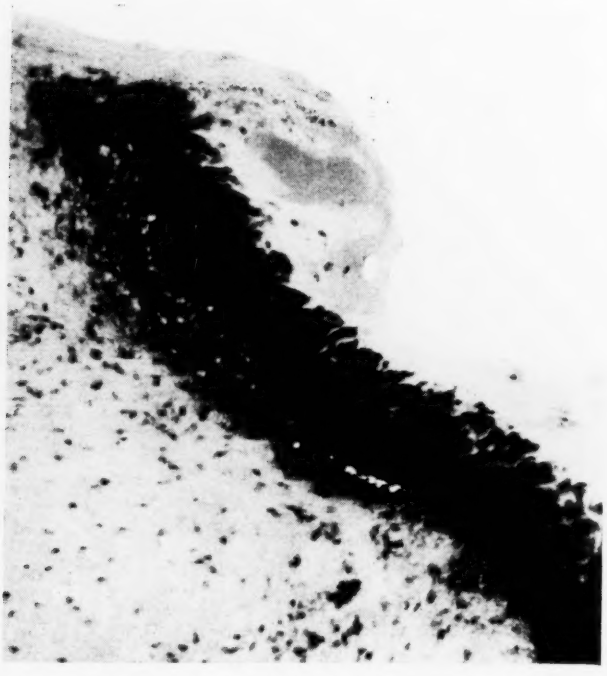

Fig. 8. — Alkaline phosphatase activity in a 16-hour vital wound. (Coupling azo dye method of Grogg and Pearse, × 100)

phosphatase activity (1 plus) were seen to have migrated out of the vessels.

In an 8-hour wound (Fig. 7) the enzyme activity demonstrable in the central zone had almost disappeared. In contrast to this the dermis in the peripheral zone showed an intensified (2 plus) staining occurring chiefly in the fibroblasts, but also in the polymorphonuclear leukocytes. The hairs and the epidermis remained unstained.

The enzyme activity in the dermis in the peripheral zone of a 16-hour wound (Fig. 8) was very strong. It was seen continually in the fibroblasts. In addition to this, mononuclear phagocytes exhibited intense (3 plus) activity. The central zone remained almost unstained, except for solitary invading leukocytes with demonstrable (1 plus) activity.

Throughout the remaining experimental period the intensity of staining of both zones was similar to that appearing in 16-hour vital wounds.

The increased enzyme activity in the peripheral zone persisted unchanged except for some diffusion at the borders, up to five days after death in skin flaps of animals killed 48 hours after wounding.

No corresponding zones were seen in the vicinity of the wounds made post mortem.

4. DISCUSSION

The staining of the uninjured skin was similar to that reported by Spier and Martin (1956). They, too, used a coupling azo dye technique. The staining of the epidermis observed by some earlier authors seemed to be an artifact caused by diffusion and absorption in the modifications of Gomori's (1939) procedure.

A slight increase in the activity could already be demonstrated four hours after the injury, but a clearly distinguishable peripheral wound zone with increased alkaline phosphatase activity was a constant feature in 8-hour vital wounds and later on. The earliest increase in the activity of this enzyme hitherto reported to occur after an injury was 24 hours (Balazs and Holmgren 1950). Previous workers usually only began their studies, however, one or two days after the wound was inflicted.

The central zone showed decreasing alkaline phosphatase activity from four hours after the injury. This diminution became more evident in 8-hour and subsequent vital wounds, but did not occur in post-mortem ones. The decrease in activity, which is apparently due to the very severe effect of the vital injury on the cells of the central area, may thus be called a negative vital reaction. The variations in the depth of the zones, showing either the negative vital reactions or an increase in activity, may be due to minor variations in the wounding process occurring in spite of every endeavour to make all the excisions in exactly the same manner.

The effect of the vital injury on the cells of the peripheral zone seemed to serve as a stimulus activating local defense mechanisms after a mobilization time of four to eight hours. The local connective tissue cells in that zone, accompanied by some invading leukocytes, were still able to produce an intense enzyme activity. This phenomenon, which did not occur in post-mortem wounds, may analogously

be called a positive vital reaction. The epidermis, being a relatively inert tissue (Needham 1952), was not able to produce such an active zone during the experimental period.

Besides functioning as a defense barrier, the alkaline phosphatase in the peripheral zone may play a role in more specific regenerative processes (Raekallio 1960). A synthetic action via transphosphorylation has been ascribed to alkaline phosphatase (Meyerhof and Green 1950, Dixon and Webb 1953, Morton 1955). Similarly, Richterich (1958) concluded, after reviewing earlier investigations, that this enzyme is in some way concerned with synthetic processes, especially with those involving fibrous protein.

Both the positive and the negative vital reactions were clearly and constantly recognizable, besides some of the preceding signs, in 8-hour vital wounds. Yet, in many cases, even the 8-hour limit may not be early enough for the medicolegal distinction between vital and post-mortem injuries. As a supplementary method the demonstration of the increased alkaline phosphatase activity in the peripheral zone and of the decreased activity in the central one might be used, however, because no false post-mortem reactions were seen and the vital activity was well preserved for five days after death.

5. SUMMARY

In the uninjured skin the epidermis showed no alkaline phosphatase activity, and after wounding it still remained unstained by the coupling azo dye technique. Increased activity, especially in the fibroblasts, could constantly be demonstrated in the peripheral dermal zone of 8-hour vital wounds (positive vital reaction). Some preceding increase was already visible there four hours after the injury, and the activity intensified up to 16 hours. In the central zone a decrease started after four hours, and the activity was still further diminished in 8-hour vital wounds (negative vital reaction). No false post-mortem staining was seen, and the vital reactions persisted clearly for five days after death. Even the 8-hour limit is not always early enough for the medicolegal distinction between vital and post-mortem wounds. However, the demonstration of the changes described might give supplementary evidence of the vital origin of a skin wound.

VI. STUDIES ON ACID PHOSPHATASE ACTIVITY

1. EARLIER INVESTIGATIONS

Non-specific acid phosphatases hydrolyze phosphomonoesters in the region of pH 5.0.

The number of references in the literature to the distribution of acid phosphatase activity is smaller than to the alkaline enzyme.

Some authors (Gomori 1941, Fisher and Glick 1947, Pirilä and Eränkö 1950) have observed no acid phosphatase activity in normal human skin, whereas others have demonstrated it in fibroblasts of rat, mouse and hamster (Noback and Paff 1951), or in the stratum granulosum of human epidermis (Mescon, Gray and Moretti 1954). Bejdl (1954) stated that in man the granular layer stained intensely, and some activity occurred in the spinous and basal layers, too. According to Spier and Martin (1956) in addition to the sites mentioned by Bejdl, a distinct staining reaction was also present in the stratum corneum and in some of the cutaneous appendages.

The acid phosphatase activity of mononuclear phagocytes has been correlated with resistance to tuberculosis (Grogg and Pearse 1952 c). The susceptible guinea-pig shows no acid phosphatase activity in these cells, in contrast to more resistant species. On the other hand, the polymorphonuclear leukocytes of the guinea-pig contain this enzyme, whereas those of the mouse and rat do not. The acquisition of this enzyme by monocytes during their transformation to macrophages has been related to their enhanced phagocytic power (Weiss and Fawcett 1953). Rosenbaum and Rolon (1960) have likewise recently pointed out the significance of acid phosphatase in phagocytosis.

Several other theories have been put forward concerning the possible roles played by acid phosphatase *in vivo*, *e.g.* in prostatic cancer (Woodard 1952, London and Hudson 1955), in the secretory

function of the kidney tubules (Eränkö and Niemi 1954), in the cytolysis of cultured cells (Green and Verney 1956), and either in increased mitochondrial activity or in actual mitochondrial damage (Pearse 1958).

According to several authors (Balazs and Holmgren 1950, Junqueira 1950, Needham 1952) acid phosphatase plays no part in wound healing and regeneration. This enzyme has been observed, however, in regenerating nerve cells (Bodian and Mellors 1945, Bodian 1947). An increase in acid phosphatase content has been stated to be a characteristic of growing tissue (Yokoyama *et al.* 1953, Büssing 1957), and the role played by this enzyme in protein synthesis has been discussed, too (Axelrod 1948, Ellis, Sewell and Skiner 1956, Vorbrodt 1958).

2. HISTOCHEMICAL METHOD

Gomori (1941) introduced a method for the histochemical demonstration of acid phosphatase analogous to his method for the alkaline enzyme, depending on the released phosphoric acid which was precipitated, and then visualized as lead sulphide. The method was criticized because of the non-specific absorption of lead by the sections (Rabinovitch, Junqueira and Fajer 1949, Newman, Kabat and Wolf 1950, Lison 1953), especially in the nuclei (Eränkö 1952).

Seligman and Manheimer (1949) applied the simultaneous coupling principle for the demonstration of acid phosphatase. Grogg and Pearse (1952 b), after criticizing the low solubility of calcium α-naphthyl phosphate used by these authors, introduced a new modification in which the sodium salt was employed as a very soluble substrate. They used fixation in cold formalin and frozen sections, in order to ensure better preservation of the enzyme. Later on (Pearse 1960), Fast garnet GBC was demonstrated to be the most suitable coupling agent in the conditions used.

According to Pearse (1960), the use of substituted naphthols as substrates (Burstone 1958), or of post-incubation coupling techniques (Rutenburg and Seligman 1955) did not aid in the histochemical demonstration of acid phosphatase. Thus the following modification of the simultaneous coupling azo dye technique by Grogg and Pearse (1952 b) was employed in this study:

Fig. 9. — Acid phosphatase activity in a 2-hour vital wound. (Coupling azo dye technique, × 100)

The procedure before incubation (fixing, freezing, cutting, mounting and drying) was exactly the same as with alkaline phosphatase.

To make the incubation mixture, 20 mg of sodium *α*-naphthyl phosphate was dissolved in 20 ml of 0.1 M-veronal acetate buffer (pH 5.0), and 20 mg of Fast garnet GBC (the diazonium salt of 4-*o*-tolyazo-*o*-toluidine) was added with shaking.

The sections were incubated at + 20°C for 40 minutes in the mixture filtered onto them, then washed in distilled water for two minutes, and mounted in glycerin jelly.

Control sections were incubated without the substrate.

3. RESULTS

The sites of acid phosphatase activity were visualized by a reddish-brown stain which was not seen in the control sections.

In the uninjured skin (Fig. 9, on the left of the picture) the stratum granulosum stained intensely (3 plus), whereas the other

Fig. 10. — Acid phosphatase activity in a 4-hour vital wound. The central zone is on the right in the picture. (Coupling azo dye technique, × 100)

epidermal strata showed a weaker (1 plus) reaction. Some activity (1 plus) was also seen in the proximal part of the hair, in the distal part of the outer root sheath, and in the sebaceous gland. A moderate (2 plus) activity was demonstrated in the inner root sheath. The fibroblasts of the dermis were also stained (1 plus).

In the injured skin no distinct changes were seen in the vicinity of 1/2-, 1-, or 2-hour (Fig. 9) wounds.

In a 4-hour vital wound (Fig. 10) the intensity of staining (1 plus) noted in the fibroblasts decreased a little in the central zone, the epidermis of which, however, retained its activity. In the peripheral wound zone an intensified (2 plus) staining reaction was seen both in the whole stratum Malpighii (except for the 3 plus activity in the stratum granulosum) of the epidermis, and in the dermis. In the latter part of the skin the increased activity chiefly occurred in the fibroblasts.

In an 8-hour wound (Fig. 11) no additional decrease in activity was seen in the central zone. In contrast to this, the staining reac-

Fig. 11. — Acid phosphatase activity in an 8-hour vital wound. (Coupling azo dye technique, × 100)

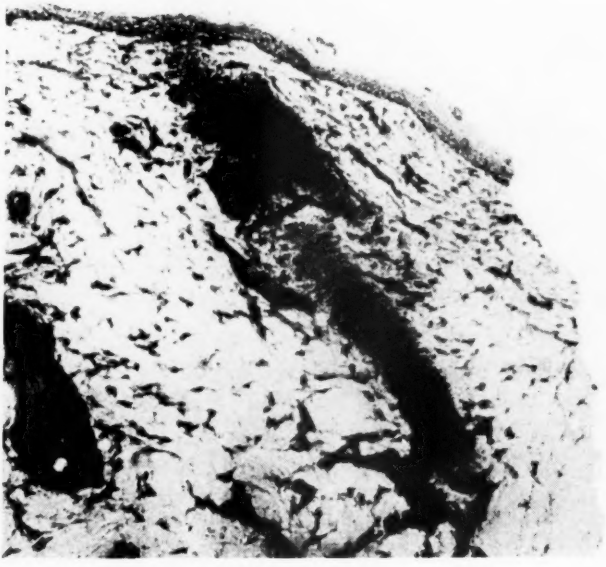

Fig. 12. — Acid phosphatase activity in an 8-hour post-mortem wound. (Coupling azo dye technique, × 100)

Fig. 13. — Acid phosphatase activity in a 16-hour vital wound. (Coupling azo dye technique, × 100)

tion in the peripheral zone became still more intense (3 plus). In addition to the fibroblasts, the polymorphonuclear leukocytes took part in this increase.

The staining retained its intensity in 16-hour wounds (Fig. 13) and later (*e.g.* in a 64-hour wound, Fig. 14) during the experimental period. The depth of the peripheral zone varied between 100 μ and 300 μ, that of the central zone between 200 μ and 500 μ.

In the skin flaps of the animals killed 48 hours after wounding vital changes in the staining intensity persisted clearly up to three days after death (*e.g.* one day after death, cf. Fig. 15). Four days post mortem (Fig. 16) a slight diffusion in the borders of

Fig. 14. — Acid phosphatase activity in a 64-hour vital wound. (Coupling azo dye technique, × 100)

the peripheral zone was observed, but even five days after death a distinct active zone could be seen.

No such zones could be demonstrated in the wounds made post mortem. Fig. 12 illustrates this in a wound made eight hours after death.

4. DISCUSSION

The distribution of acid phosphatase activity in the uninjured skin was similar to that observed by Spier and Martin (1956). They, too, used a coupling azo dye technique. The absence of acid phosphatase activity in the epidermis, as well as the lack

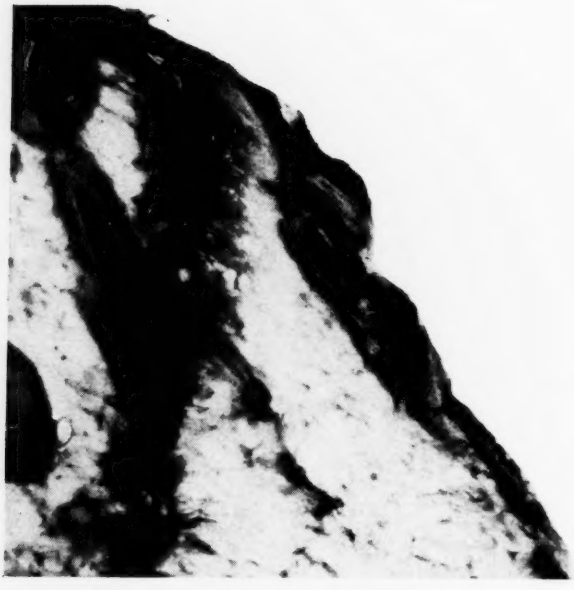

Fig. 15. — Acid phosphatase activity in a 48-hour vital wound. The sample was removed from the animal one day after death in order to study the post-mortem stainability. (Coupling azo dye technique, × 100)

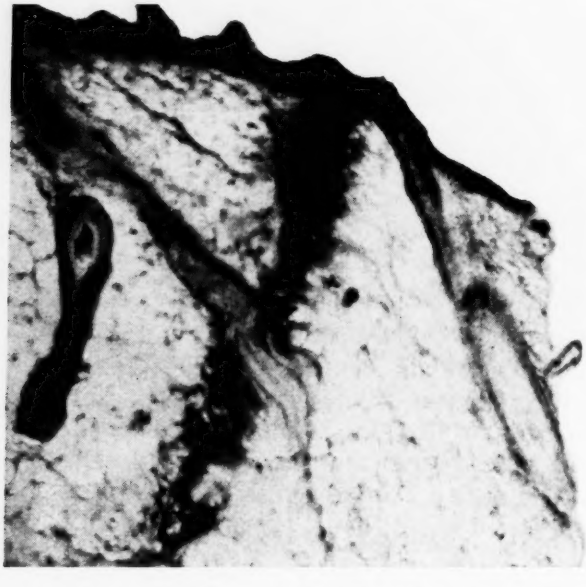

Fig. 16. — Acid phosphatase activity in a 48-hour vital wound. The sample was removed from the animal four days after death. (Coupling azo dye technique, × 100)

of demonstrable changes in wound tissue reported by many earlier authors, may be due to the shortcomings of Gomori's technique. For instance, only 10 per cent of acid phosphatase remained in the tissues after paraffin embedding (Taft and Scott 1958), as compared with the 55 per cent after fixation in cold formalin and freezing (Seligman, Chauncey and Nachlas 1951).

The significance of acid phosphatase in the peripheral zone of a healing wound might be the same as was discussed in connection with the alkaline enzyme. The increase in acid phosphatase activity, however, represents an earlier response to the stimulating effect of an injury. Here, too, acting synthetically, the enzyme might, by transphosphorylation, play a reparative role from early in wound healing.

The opinion has been advanced, however, that hydrolases hardly ever have a synthetic or transferring action (de Duve 1959). Moreover, there is in fact both room and need for much purely hydrolytic activity in the functions of defensive cells. Hydrolytic breakdown of cell constitutients, and phagocytosis might be of special importance in the process of wound healing. The intensification of acid phosphatase activity some time before polymorphonuclear leukocytes or mononuclear phagocytes appear in the peripheral wound zone, is in keeping with the view (de Duve 1959) that phagocytic properties are not restricted to a few cell types. For instance fibroblasts might play a part in this process, alongside the other cells mentioned.

There was a minimal negative vital reaction in the central zone from four hours after the injury. This was compatible with the reports (Chang *et al.* 1958, Stowell, Chang and Berenbom 1961) that acid phosphatase in tissue undergoing progressive necrosis showed a later and less marked loss of activity than the alkaline enzyme. By contrast, the positive vital reaction appeared at that time as a clearly distinguishable increase of acid phosphatase activity in the peripheral zone. The 4-hour limit is already of considerable value for medicolegal purposes. The validity of this test is accentuated by the fact that no false positive post-mortem reactions were seen, and that the vital changes in activity could be demonstrated for five days after death. The possibility of using fixed tissue specimens is a very important point if the method is to be introduced as a practicable medicolegal test.

5. SUMMARY

In the uninjured skin the stratum granulosum showed a strong acid phosphatase activity and some staining was also observed in the other epidermal layers, in some cutaneous appendages, and in the fibroblasts. In the injured skin the peripheral wound zone, consisting of the stratum Malpighii in the epidermis, and chiefly of fibroblasts in the dermis, exhibited an intensified acid phosphatase activity beginning in 4-hour vital wounds and increasing up to eight hours (positive vital reaction). In the central zone a minor decrease in staining intensity was observed from four hours after injury (negative vital reaction). The vital reactions persisted fairly clearly for five days after death, and no false positive staining was seen post mortem. This confirmed the conclusion that the demonstration of zones showing these changes in acid phosphatase activity might be of considerable value in the medicolegal distinction between vital and post-mortem skin wounds.

VII. STUDIES ON AMINOPEPTIDASE ACTIVITY

1. EARLIER INVESTIGATIONS

Peptidases are enzymes capable of hydrolyzing peptide linkages. Exopeptidases are limited in their action to -CO-NH- bonds adjacent to a free α-carboxyl or α-amino group in the substrate, whereas endopeptidases attack peptide linkages not close to these groups (Fruton 1946). Aminopeptidases belonging in the former category hydrolyze a variety of peptides which have a free α-amino group on a terminal leucine or other related amino acid. Chemically »leucine» aminopeptidase is considered to be a simple protein (Smith 1951, Spackman, Smith and Brown 1955, Smith and Spackman 1955). It has been suggested, however, that »leucine» aminopeptidase is a mixture of peptidases, and does not represent a single enzyme (Waldschmidt-Leitz and Keller 1957, Glenner, Burstone and Meyer 1959). For that reason the less specific and shorter term, aminopeptidase, will be used in this study.

There are few investigations on the distribution of aminopeptidase in the skin. Opinions differ as to whether the stratum basale of human epidermis exhibits (Braun-Falco 1957 a, b) activity of the enzyme or not (Nachlas *et al.* 1960). Fibroblasts, polymorphonuclear leukocytes, sweat glands, and the adventitia of most blood vessels have been observed to give an intense staining reaction (Nachlas *et al.* 1960).

Several hypotheses have been put forward to explain the occurrence of aminopeptidase in various physiological and pathological conditions. The enzyme has been related either to synthesis or to hydrolysis of parathyroid hormone (Pearse and Tremblay 1958), to secretion of some agent of a protein nature by the pineal gland (Niemi and Ikonen 1960), to liberation of heparin from its lipoprotein complex in mast cells (Braun-Falco and Salfeld 1959),

or to invasiveness of tumours (Sylvén and Malmgren 1955, Burstone 1956, Braun-Falco 1957 b, c, Glenner, Burstone and Meyer 1959). The view has been presented, however, that the aminopeptidase activity of the stroma of neoplasms is a biological property of proliferating connective tissue cells, and that it bears no special correlation to the invasiveness of malignant tumours (Monis, Nachlas and Seligman 1959). In wound tissue the presence of proteolytic enzymes has been demonstrated biochemically (Wells and Babdock 1953, Ungar and Damgaard 1954, Howes, Armitage and Mandl 1955). There have been only a few histochemical studies on aminopeptidase activity in healing wounds. According to Glenner, Burstone and Meyer (1959), fibroblasts of human wounds stain minimally, whereas macrophagic and inflammatory cells show an intense reaction. By contrast, Monis, Nachlas and Seligman (1959) have observed strong aminopeptidase activity in the proliferating fibroblasts of 3-day rat skin wounds. Seven days after injury, rows of intensely active fibroblasts dominate the picture, in addition to the staining reaction demonstrable in macrophages and polymorphonuclear leukocytes. According to these workers, cultured fibroblasts also show a very strong aminopeptidase activity.

2. HISTOCHEMICAL METHOD

Gomori (1954) developed the first histochemical method for the demonstration of aminopeptidase activity, too. He modified the coupling azo principle, employing chloroacetylnaphthylamines as substrates and Fast garnet GBC as diazonium salt. Burstone and Folk (1956) obtained a better localization with L-leucyl-β-naphthylamide as substrate for the enzyme which is activated by cyanide in low concentrations. Nachlas, Crawford and Seligman (1957) used the same substrate, but another coupling agent, Fast blue B. The azo dye produced does not give large crystals like the former diazotate, and is able to form a stable copper chelate, which is deep bluish-purple in colour. Thus, in spite of its considerable inhibitory effect (60 per cent, as compared with 30 per cent by Fast garnet GBC, Nachlas, Crawford and Seligman 1957) Fast blue B seems to be more satisfactory as the diazonium salt. Furthermore a new substrate, coupling more rapidly and thus preventing

diffusion, has been synthesized (Rosenblatt, Nachlas and Seligman 1958), and succesfully used for the histochemical demonstration of the enzyme (Nachlas *et al.* 1960). Since this substrate, L-leucyl-4-methoxy-β-naphthylamide, was not available, the following modification of the original method described by Nachlas, Crawford and Seligman (1957) was used in this study:

The stock substrate solution was prepared by dissolving L-leucyl-β-naphthylamide (Dajac Laboratories, The Borden Chemical Company) in distilled water at a concentration of 8 mg per ml. The incubation medium contained the following reagents:

Stock substrate solution	1 ml
Acetate buffer (0.1 M, pH 6.5)	10 ml
Sodium chloride solution (0.85 per cent)	8 ml
Potassium cyanide solution (2×10^{-2} M)	1 ml
Fast blue B (the diazonium salt of *o*-dianisidine)	10 mg

The Procedure: 1. Unfixed halves of skin flaps were rapidly frozen with solid carbon dioxide.

2. Sections were cut at 15 μ with a rotary microtome in a cryostat at — 20°C, picked up on warm coverslips, thawed, and allowed to dry at room temperature for five minutes.

3. This was followed by incubation at + 37°C for two hours.

4. The sections were rinsed in saline for two minutes, then immersed in 0.1 M-cupric sulphate in 5 per cent neutral formalin for 30 minutes, rinsed again in saline (0.85 per cent sodium chloride solution), and mounted in glycerin jelly. Control sections were incubated without the substrate.

3. RESULTS

In the uninjured skin a distinct (1 plus) activity could be shown in the fibroblasts both of the connective tissue sheaths of active hair follicles, and of the dermis. At the last-named site

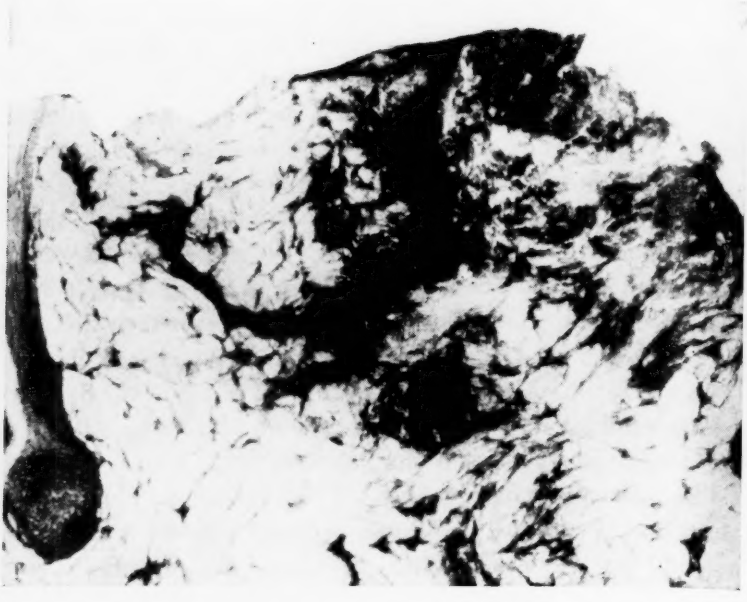

Fig. 17. — Aminopeptidase activity in a 1-hour vital wound. (Modification of the method of Nachlas, Crawford and Seligman, × 100)

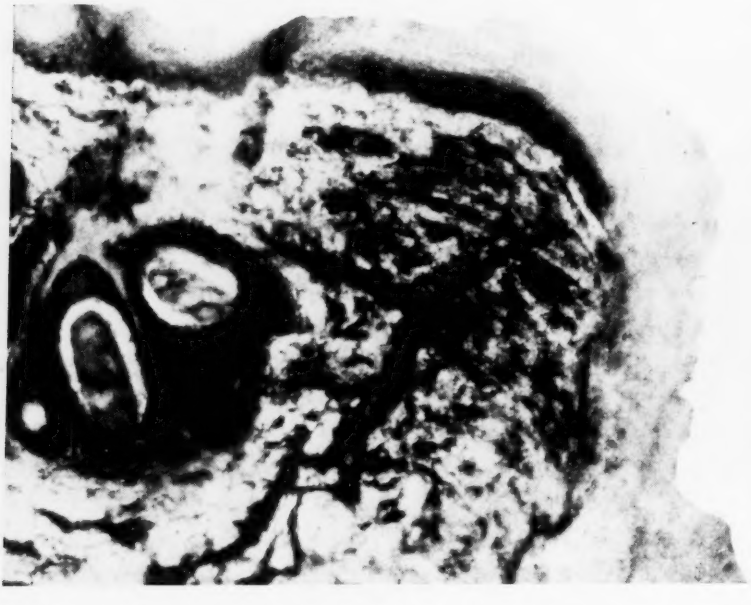

Fig. 18. — Aminopeptidase activity in a 2-hour vital wound. (Modification of the method of Nachlas, Crawford and Seligman, × 100)

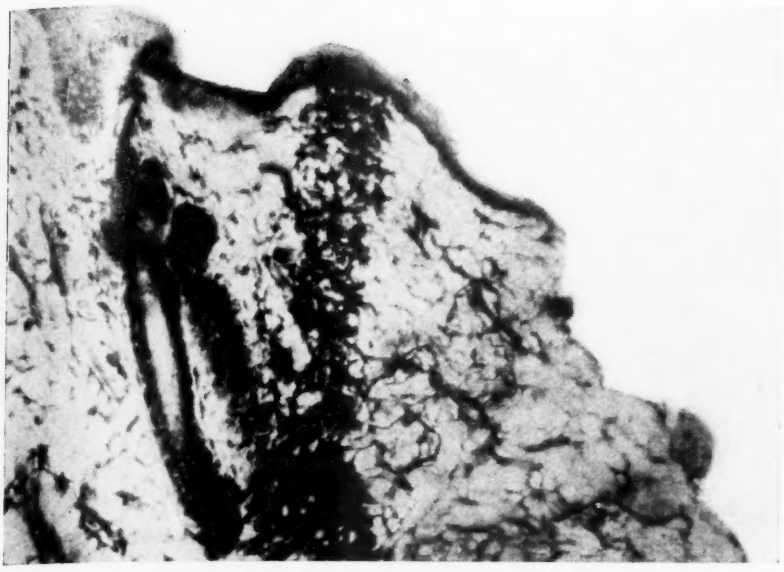

Fig. 19. — Aminopeptidase activity in a 16-hour vital wound. (Modification of the method of Nachlas, Crawford and Seligman, × 100)

the fibroblasts seemed to form a syncytial network (Fig. 17, on the left in the picture) by contact between their delicate cell processes. A moderate (2 plus) activity was seen in the basal and spinous layers of the epidermis, an in the outer root sheaths of active hair follicles.

No distinct changes usually occurred in 1/2- or 1-hour wounds. In some cases, however, a diffuse increase (2 plus) was seen in the stainability of fibroblasts in the vicinity of a 1-hour vital wound (Fig. 17, on the right in the picture).

In a 2-hour vital wound (Fig. 18) a peripheral zone showing an intensified (2 plus) aminopeptidase activity of the fibroblasts was regularly observed. At the same time a slight decrease in the stainability of the connective tissue cells sometimes occurred (Fig. 18) in the central zone.

In 4- and 8-hour wounds aminopeptidase activity in the peripheral zone increased very little beyond that (2 plus) observed two hours after injury. The fibroblasts seemed continually to play the leading role among the cells forming the active peripheral zone, but polymorphonuclear leukocytes participated in it when

Fig. 20. — Aminopeptidase activity in a 32-hour vital wound. The central wound zone, followed by the peripheral one, is on the right in the picture. (Modification of the method of Nachlas, Crawford and Seligman, × 100)

eight hours had elapsed after the injury. In the central zone the activity (1 plus or less) demonstrable in 2-hour wounds persisted during the remaining experimental period.

In 16-hour wounds the staining reaction became still more (3 plus) intense in the fibroblasts and in the mononuclear phagocytes of the peripheral zone (Fig. 19). No further increase in aminopeptidase activity could be seen after that time (*e.g.* in a 32-hour wound, cf. Fig. 20).

The peripheral zone was initially slightly diffuse at the borders and up to 400 μ deep (Fig. 18), but later on it became more restricted, its depth then varying between 100 μ and 300 μ (*e.g.* 16 hours after wounding, cf. Fig. 19), and that of the central zone being between 200 μ and 500 μ.

Broadly speaking, the location and intensity of staining of the zones persisted unchanged up to five days after death. A slight diffusion was seen at the margins after the first post-mortem day.

In the neighbourhood of the wounds made post mortem no such zones could be demonstrated (*e.g.* 32 hours after death, cf. Fig. 21).

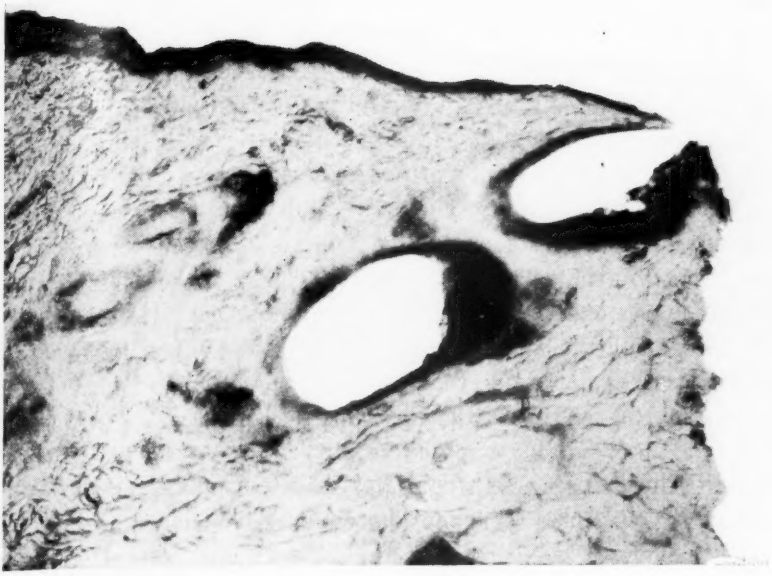

Fig. 21. — Aminopeptidase activity in a 32-hour post-mortem wound. The wound edge is on the right in the picture. (Modification of the method of Nachlas, Crawford and Seligman, × 100)

4. DISCUSSION

According to Nachlas *et al.* (1960) their new substrate gives no significantly different localization of the enzyme in tissues as compared with the results obtained with L-leucyl-β-naphthylamide. The writers could not, however, demonstrate the activity observed in the lower layers of the stratum Malpighii by Braun-Falco (1957 a, b), and in the present study. Whether the staining in that part of the skin is an artifact must remain an open question until a reinvestigation making use of both substrates can be carried out.

With both of them, the distinct enzyme activity of fibroblasts has been shown (Monis, Nachlas and Seligman 1959, Nachlas *et al.* 1960). In this connection it is necessary to discuss the basic role of these cells in increasing the activities of hydrolytic enzymes, such as phosphatases and aminopeptidase, in wound tissue.

According to Montagna (1956), the fibroblast is the master cell of connective tissue. This cell must be delicately balanced and attuned to the biological demands of the organ. The fibroblast

undergoes countless modifications which represent the imprints left upon it by changes in the environment.

Thus it is not surprising that the fibroblasts respond to injury by producing strong enzyme activities in the peripheral zone of the wound. Even foreign body granulomata (Gedigk and Bontke 1957) have been shown to provoke mesenchymal cells to display an increase in hydrolytic enzyme activity. It now seems that a wound likewise serves as such a stimulus. In the central zone, the destruction of local connective tissue cells prevents them reacting positively, as compared with the stimulating action of the injury on the peripheral zone. The latter area shows intensified enzyme activities long before a distinct leukocytic zone is visible.

In the central zone there was only a minimal negative vital reaction, *i.e.* slightly decreased activity from two hours after the injury. Thus, like acid phosphatase, aminopeptidase seemed to be less sensitive than alkaline phosphatase to the phenomena connected with progressive necrosis. On the other hand, a positive vital reaction, *i.e.* an increase in the aminopeptidase activity invariably appeared in the peripheral zone of wounds at least 2 hours old. From the medicolegal point of view, this is of importance, all the more so because the vital changes in activity could be demonstrated for five days after death and because no false post-mortem reactions were observed.

5. SUMMARY

The lower layers of the stratum Malpighii, some parts of the active hair follicles, and the fibroblasts showed aminopeptidase activity in the uninjured skin. From two hours after the vital injury, increased activity (a positive vital reaction) could be demonstrated in the peripheral wound zone, which consisted chiefly of dermal fibroblasts. The staining reaction intensified up to 16 hours. In the central zone the activity slightly decreased from two hours after wounding (negative vital reaction). No false post-mortem staining reactions were seen and the vital changes were fairly well preserved for five days after death. The two-hour limit is the earliest one observed by the methods used in this study for the distinction between vital and post-mortem skin wounds.

VIII. STUDIES ON CYTOCHROME OXIDASE ACTIVITY

1. EARLIER INVESTIGATIONS

Cytochrome oxidase enables aerobic cells to utilize molecular oxygen for the oxidation of intermediary metabolites to carbon dioxide and water (Hoffman, Rottino and Stern 1951). The cytochrome system functions as a biological electron-transporting mechanism, for instance in the oxidative reactions of the Krebs' cycle, and consists of three parts: (1) the cytochrome pigments (a, b, c), (2) the cytochrome reductases, and (3) cytochrome oxidase, an enzyme which catalyzes the oxidation of the reduced cytochrome pigments (Lerner 1954, Leonhardi 1959).

The investigation of cytochrome system proceeded along three lines. In 1886, MacMunn discovered the presence of respiratory pigments which he called myo- and histohaematins. The significance of this observation was overlooked until Keilin (1925) reaffirmed MacMunn's work, and suggested the name cytochrome. Later on, a distinction was made between cytochrome a_1, a_2 and a_3 (Keilin and Hartree 1938, 1939).

Warburg and Negelein (1929) gave the name »Atmungsferment» to the iron containing enzyme involved in cellular respiration.

Ehrlich (1885) injected a mixture of alpha naphthol and N,N-dimethyl-*p*-phenylenediamine into animals, causing the formation of indophenol blue (the Nadi reaction). The catalytic effect of the living tissue was attributed to a special enzyme, named indophenol oxidase (Schultze 1909).

The identity of cytochrome a_3, »Atmungsferment», indophenol oxidase, and cytochrome oxidase was later established (Keilin and Hartree 1938, 1939), and the enzyme was purified (Smith and Stotz 1954).

In the skin, the maximal accumulation of indophenol blue granules has been observed in the matrix of the hair bulb, and some reaction has also been detected in the proximal third of the outer root sheath (Montagna 1956). Opinions differ as to whether the staining in the stratum Malpighii of the epidermis is non-granular and only light blue (in sheep skin, Rogers 1953), or whether the basal layers of the epidermis show an intense reaction (in man, Steigleder 1957).

Leukocytes have been listed in the order of decreasing cytochrome oxidase activity as follows: monocytes, macrophages, lymphocytes, and polymorphonuclear leukocytes (Hoffman, Rottino and Stern 1951). The Nadi reaction in the neutrophils and lymphocytes of inflammatory exudates has been observed to be distinctly stronger than that in the cells of the blood (Rebuck 1952, Wachstein 1955). On the other hand, peroxidase activity has been claimed to be, at least partly, responsible for the Nadi reaction demonstrable in leukocytes (Hannibal, Nachlas and Seligman 1960).

An initial decrease in cytochrome oxidase content, followed by a return to normal values after three or four days, has been observed in biochemical studies on regenerating liver (Novikoff and Potter 1948). In a histochemical study, however, an intensified Nadi reaction has been demonstrated prior to the occurrence of cell divisions in wounded plant tissues (Baba 1953). According to Tonna (1959), cytochrome oxidase activity likewise increases in the periosteum of injured bone in the rat. Seven days after the operation, all the cells at the site of the trauma reveal the staining reaction, which is most intense at the outermost edges of the callus tissue.

2. HISTOCHEMICAL METHOD

Gräff (1916) adapted Ehrlich's (1885) mixture for histochemical use in the so-called labile or G (»Gewebe»)-Nadi reaction. Many authorities, however, pointed out the occurrence of diffusion artifacts due to the lipophilia of indophenol blue (Glick 1949, Gomori 1953, Nachlas *et al.* 1958). Furthermore, the reactions proceeded spontaneously in the presence of oxygen (Nachlas *et al.* 1958).

Nachlas *et al.* (1958) modified the Nadi reaction by replacing the classical amine with a new one. Unfortunately the resulting dye was also lipid soluble (Pearse 1960). Burstone (1959) tested a number of amines in place of the original one. He found that N-phenyl-*p*-phenylenediamine exhibited a low rate of auto-oxidation, and, after making experiments with a series of substituted naphthols, observed that 1-hydroxy-2-naphthoic acid, for instance, was a very useful coupling component. Post-fixation and complexing with a metal prevented any decolourization due to the action of reducing sites in the tissues, and darkened the colour.

The following version of Burstone's (1959) method was used in this study:

The incubation solution was prepared by dissolving 10 mg of 1-hydroxy-2-naphthoic acid (Fluka AG) and 10 mg of N-phenyl-*p*-phenylenediamine (Fluka AG) in 0.5 ml of reagent ethanol. Thereafter, 35 ml of distilled water and 15 ml of 0.2 M-»Tris» buffer (pH 7.2) were added. The solution was shaken and filtered into a Coplin jar.

The procedure before incubation was that described in connection with the method for aminopeptidase (freezing of unfixed specimens, followed by cutting, picking up, thawing, and drying of the sections).

The 15 μ thick sections were incubated at + 37°C for two hours. The slides were then transferred to a 10 per cent solution of cobalt acetate in 10 per cent neutral buffered formalin for one hour. After this post-fixation and complexing with cobalt, the preparations were washed in distilled water, and mounted in glycerin jelly.

Control sections were immersed in a solution of the specific (Moog 1943) inhibitor of the enzyme (0.005 M-sodium azide in 0.85 per cent saline) for two minutes before incubation.

3. RESULTS

Cytochrome oxidase activity appeared as a brownish-black cytoplasmic staining. Control sections were negative.

In the uninjured skin, a moderate (2 plus) reaction was seen in the stratum basale and in the lower cells of the stratum spinosum. The intensity of staining diminished (1 plus) in the cells higher up

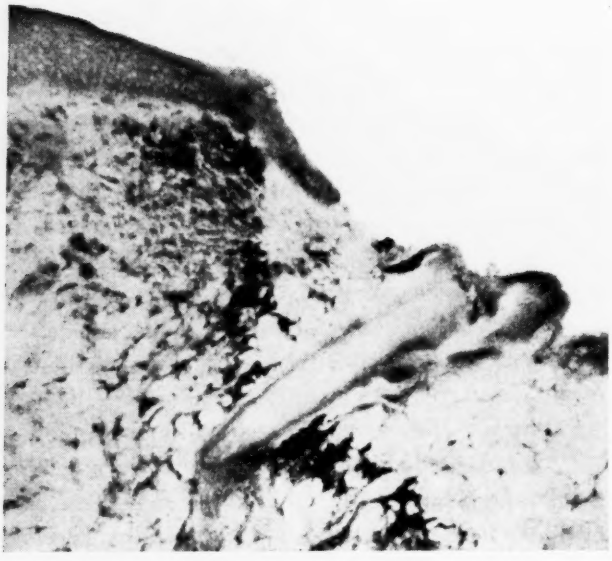

Fig. 22. — Cytochrome oxidase activity in an 8-hour vital wound. (Modification of the method of Burstone, × 100)

in the latter layer (Fig. 22 and 23, on the left of the pictures). The stratum granulosum and stratum corneum showed no cytochrome oxidase activity. The peripheral cells of the sebaceous glands reacted strongly (3 plus). In active hair follicles a moderate (2 plus) enzyme activity appeared in the matrix, in the dermal papilla, and in the root sheaths. The fibroblasts of the dermis reacted, too (1 plus to 2 plus).

In the injured skin no distinct changes were seen in 1/2-, 1- or 2-hour wounds. Four hours after the vital injury the stainability of the fibroblasts in the central zone began to decrease (1 plus or less).

In an 8-hour vital wound (Fig. 22) a distinct peripheral zone was observed showing increased (2 plus) activity of the fibroblasts and of some migrated polymorphonuclear leukocytes. At the same time, the central zone exhibited almost no cytochrome oxidase activity, except in the lower cells of the epidermis, and in the root sheaths of the hair follicles (Fig. 22, on the right in the picture).

16 hours after the vital injury the picture was essentially the same observed in 8-hour vital wounds. Invading mononuclear

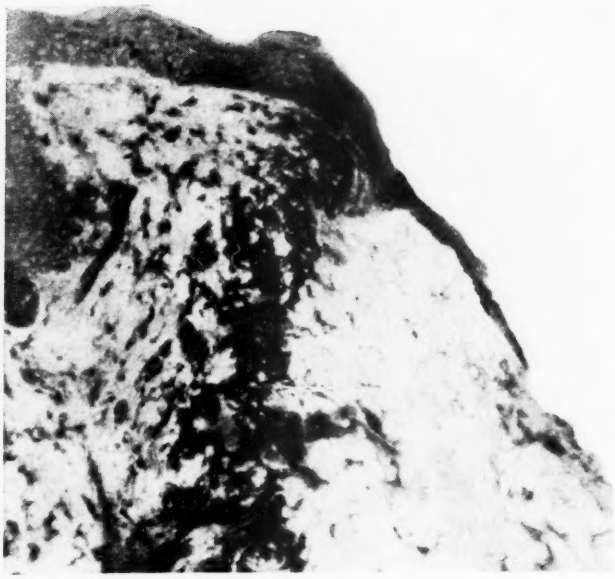

Fig. 23. — Cytochrome oxidase activity in a 32-hour vital wound. (Modification of the method of Burstone, × 100)

phagocytes were beginning, however, to take part in the formation of the active peripheral zone.

In 32-hour vital wounds (Fig. 23) the fibroblasts of the central zone continued to show practically no staining reaction. By contrast, in the peripheral zone these cells reacted intensely (3 plus), as did likewise the lower cells of the epidermis. Mononuclear phagocytes participated in the increased cytochrome oxidase activity observed in the dermis of the peripheral zone. No additional intensification of the staining reaction was observed in 64- and 128-hour wounds.

The depth of the peripheral zone varied, after its first appearance, between 100 μ and 300 μ, that of the central zone between 200 μ and 500 μ.

A moderate diffusion was seen on the margins of the peripheral zone from the first post-mortem day, but the reported vital changes in cytochrome oxidase activity were recognizable up to five days after death.

No such zones could be demonstrated in the vicinity of the wounds made post mortem.

4. DISCUSSION

The distribution of cytochrome oxidase activity in the skin with its appendages was largely similar to that observed by Steigleder (1957), who used the Nadi reaction. Incubation in Burstone's mixture, however, resulted in a more definite localization of the enzyme.

The aerobic oxidation of intermediary metabolites to carbon dioxide and water produces approximately fifteen times as much energy as anaerobic glycolysis (Lerner 1954). The presence of cytochrome oxidase, as was mentioned, makes the utilization of molecular oxygen possible in the former process. This may explain the strong activity of this enzyme observed in the cells with a high energy requirement, *e.g.* in those of the proliferating lower part of the epidermis.

On the other hand, in the early stages of regeneration (Novikoff and Potter 1948) anaerobic glycolysis, being more compatible with the relatively acid local reaction, replaces aerobic oxidation (Needham 1952). Likewise, the anaerobic glycolysis of neoplastic tissues is considered to be a sign of primitiveness indicating a phylogenetically older mode of producing energy (Richterich 1958). Further it has been stated that, in regard to energy production, the cells at the onset of the cancer are identical with those in the process of wound healing (Berglas 1959).

There is, therefore, no reason to expect a sudden increase in cytochrome oxidase activity to be demonstrable in the injured skin. On the other hand, the relatively early decrease in the activity of this enzyme in the central wound zone seems to be one of the initial signs of the progressive necrosis occurring there. This is in conformity with the earlier investigations, (Stowell, Berenbom and Chang 1954), which showed that it is the oxidative enzymes which decrease most rapidly during cell death.

From the medicolegal point of view, the 8-hour limit for the onset of the positive vital reaction, and the 4- to 8-hour period before a clearly distinguishable negative vital reaction could be detected offer no advantages, as compared with the results obtained by the methods for the hydrolytic enzyme studies. The demonstration of the changes described might, however, provide supplementary evidence of the vital origin of a wound.

5. SUMMARY

The lower part of the epidermis, some cutaneous appendages, and the fibroblasts exhibited cytochrome oxidase activity in the uninjured skin. In 4-hour vital wounds the staining of the fibroblasts in the central zone decreased, and the loss became more obvious eight hours after the injury (negative vital reaction). An increased activity (positive vital reaction) could be demonstrated in the peripheral zone of 8-hour vital wounds, intensifying up to 32 hours. The active peripheral zone consisted of fibroblasts and migrated cells in the dermis, and, later on, also of basal and spinous cells in the epidermis. The vital changes were recognizable up to five days after death, and no false positive post-mortem staining reactions were seen. The histochemical demonstration of cytochrome oxidase activity is of supplementary value only in the medicolegal distinction between vital and post-mortem skin wounds.

IX. STUDIES ON SUCCINATE DEHYDROGENASE ACTIVITY

1. EARLIER INVESTIGATIONS

Dehydrogenases catalyze the transfer of hydrogen to immediate acceptors other than oxygen (Wachstein 1955). In the Krebs' cycle succinate is oxidized to fumarate by a specific enzyme, succinate dehydrogenase. The latter name, referring to the substrate, will be used in this study. According to Pearse (1960) many earlier authors have been cautious in interpreting their results as due to the specific enzyme, preferring the term succinic dehydrogenase or succinic oxidase system.

The histochemical distribution of succinate dehydrogenase has been studied in the skin of the rat (Padykula 1952, Steigleder 1955), sheep (Rogers 1953), guinea-pig (Formisano and Montagna 1954), and man (Montagna and Formisano 1955, Steigleder 1955, Foraker and Wingo 1955). The authors have observed the strongest reaction in the stratum basale, the activity diminishing in the stratum spinosum. The eccrine sweat glands, the hair matrix, and the dermal papillae are chatacterized by an intense colour reaction, and some activity has been showed in the sebaceous glands, and in the root sheaths, too.

Several theories exist concerning the possible role played by succinate dehydrogenase *in vivo*. It has been related to absorptive or secretory activities (Padykula 1952, Mustakallio 1956), to mitoses (Bullough 1952, Argyris 1956 a), or to growth in general (Foraker, Denham and Celi 1954). Whatever its most important function, succinate dehydrogenase is one of the enzymes involved in the Krebs' cycle (tricarboxylic acid cycle) (Lerner 1954, Niemi,

Siurala and Sundberg 1960, and others). Thus, it plays a vital role in the respiratory processes of living cells (Leuthardt 1959).

According to Argyris (1956 b) succinate dehydrogenase activity increases in 4-day wounds of mouse skin. The granulation tissue displays an intense staining reaction which is also seen in the basal layers of the thickened epidermis adjacent to the wound. The injured hair follicles have lost their organization, and appear as cords of enlarged cells showing elevated activity of this enzyme.

2. HISTOCHEMICAL METHOD

The histochemical demonstration of dehydrogenases is based on their ability to reduce colourless tetrazolium salts to highly coloured, water-insoluble formazans (Kuhn and Jerchel 1941, Nineham 1955). Among the disadvantages of the tetrazoles most frequently used in histochemistry, such as triphenyl tetrazolium chloride (Straus, Cheronis and Straus 1948, Kun and Abood 1949), neotetrazolium (Antopol, Glaubach and Goldman 1948), blue tetrazolium (Rutenburg, Gofstein and Seligman 1950), and the iododerivate INT (Fox and Atkinson 1950), are their lipid solubility, tendency to crystallize in tissue sections and insensitiveness (Nachlas *et al.* 1957). Therefore, efforts have been made to synthesize new compounds (Tsou *et al.* 1956). A *p*-nitrophenyl substituted ditetrazole, Nitro-BT, has been used by Nachlas *et al.* (1957). According to these workers, the dinitroformazan produced is insoluble in lipids, and initially crystals are not formed in the tissues. Dehydration in alcohol solutions may, however, cause some crystallization (Pearse 1960). According to polarographic measurements (Kivalo and Mustakallio 1956, Pearse 1960) Nitro-BT seems to have the highest oxidizing capacity among the tetrazolium compounds studied. Thus, being able to compete successfully with oxygen for electrons, this ditetrazole is a very sensitive indicator of succinate dehydrogenase activity.

In view of these facts, the following modification of the method by Nachlas *et al.* (1957) was used:

The stock buffered succinate solution was prepared by combining 5 ml of 0.2 M-sodium succinate and 5 ml of 0.2 M-phosphate buffer (pH 7.6). To make the incubation medium 10 ml of stock

solution was added to 10 ml of aqueous solution of Nitro-BT (1 mg/ml). The tetrazolium salt, 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride, was received from the Dajac Laboratories of the Borden Chemical Company.

The procedure consisted of the following steps:

1. Unfixed halves of skin flaps were rapidly frozen with the aid of solid carbon dioxide.
2. Sections were cut at 8 μ with a rotary microtome in a cryostat at — 20°C, picked up on warm coverlips, thawed, and allowed to dry at room temperature for about five seconds.
3. The sections were incubated at + 37°C for one hour, post-fixed in 10 per cent neutral buffered formalin for five minutes, washed in distilled water, and mounted in glycerin jelly.

Control sections were incubated without sodium succinate.

3. RESULTS

Succinate dehydrogenase activity appeared as a blue cytoplasmic staining. Some reddish reaction product was also seen, but it was easily distinguishable from the blue colour. Control sections were negative.

The distribution of succinate dehydrogenase activity in the uninjured skin (Fig. 24) was roughly the same as that of cytochrome oxidase (2 plus in the basal and lower spinous cells of the epidermis, in the matrix, dermal papilla, and the root sheaths of the active hair follicles, 3 plus in the peripheral cells of the sebaceous glands). Dermal fibroblasts gave a weaker staining reaction (1 plus or less).

In the injured skin no recognizable changes were seen in 1/2-, 1- and 2-hour wounds. After four hours the succinate dehydrogenase activity of the fibroblasts decreased in the central zone where these cells showed a scarcely discernible staining reaction.

In an 8-hour vital wound the central zone exhibited almost no succinate dehydrogenase activity. In the peripheral zone the

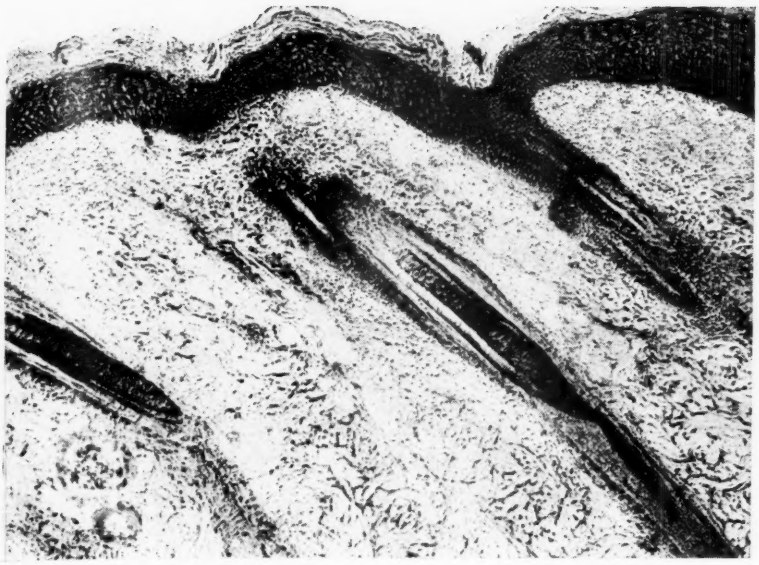

Fig. 24. — Succinate dehydrogenase activity in guinea-pig skin. (Modification of the Nitro-BT-method of Nachlas *et al.*, × 100)

fibroblasts and some invading leukocytes stained a little deeper (1 plus) than in the uninjured skin. The slight intensification of stainability could not be recorded photographically.

In 16- and 32-hour vital wounds there was a small additional increase (1 plus or little more) in the activity of the 100 μ to 300 μ deep peripheral zone, consisting mainly of fibroblasts and of mononuclear phagocytes. No activity was seen in the central zone, the depth of which varied between 200 μ and 500 μ.

The stainability of the peripheral zone did not intensify additionally in 64- and 128-hour vital wounds. 128 hours after the injury, the 2-plus reaction, characteristic of the lower cells of the stratum Malpighii, was seen in the proliferating basal layers of the thickened epidermis adjacent to the wound.

The staining reaction was already considerably diffused one day after death, but the vital increases and decreases in succinate dehydrogenase activity were discernible up to five days post mortem.

No such changes were seen adjacent to wounds made after death.

4. DISCUSSION

The appearance of two different coloured products, one blue and the other reddish, from Nitro-BT is a complication in the use of this ditetrazole. The reddish stain, a monoformazan, has been claimed to occur in areas of lower enzyme activity where a reduction of the ditetrazole has taken place at one end of the molecule only (Montagna 1956). According to another explanation (Burtner, Bahn and Longley 1956), the red derivative is probably the formazan from a contaminating monotetrazole.

The present study confirms the results of several previous workers (Rogers 1953, Montagna 1956, Steigleder 1959), who have histochemically demonstrated the identical distribution of cytochrome oxidase and succinate dehydrogenase activities in the skin. This similarity accentuates the prime importance of the latter enzyme, too, to cells with a high energy requirement. It was therefore surprising to observe the weak increase in succinate dehydrogenase activity in the vicinity of vital wounds, where intense cytochrome oxidase activities could be demonstrated. This discrepance might be due to the tetrazolium salt used in the histochemical method for succinate dehydrogenase. Tetrazolium salts may cause an inhibition of enzyme activity, and they are, in fact, highly toxic, particularly to fibroblasts (Rutenburg, Gofstein and Seligman 1950, Pearse 1960). These are the cells that produce high activities of enzymes in the peripheral wound zone in response to injury.

The negative vital reaction, *i.e.* a decrease in succinate dehydrogenase activity in the central zone detectable from four hours after injury, began simultaneously with that demonstrable by the method for cytochrome oxidase. So did the positive vital reaction, an increase in the enzyme activity in the peripheral zone discernible eight hours after wounding, but its weakness reduces the medicolegal significance of the results obtained by the histochemical method for succinate dehydrogenase.

5. SUMMARY

In the uninjured skin the distribution of succinate dehydrogenase activity was similar to that observed with the method

for cytochrome oxidase. Four hours after a vital injury the stainability of fibroblasts decreased in the central zone, and no activity was observable there after eight hours (negative vital reaction). At that time succinate dehydrogenase activity increased a little in the peripheral zone. The staining reaction showed an additional minor intensification in 16- and 32-hour vital wounds, a positive vital reaction. No false post-mortem reactions were seen and the vital changes were discernible for five days after death. Because of the relatively slight positive vital reaction demonstrable by the histochemical method for succinite dehydrogenase, this technique is less useful for the distinction between vital and post-mortem skin wounds.

X. STUDIES ON NUCLEIC ACIDS

1. EARLIER INVESTIGATIONS

The nucleic acids present in tissues in combination with basic proteins, are highly polymerized polynucleotides. They consist of purine and pyrimidine bases, phosphoric acid, and either a pentose or a desoxypentose sugar. The nucleic acid containing pentose is usually called ribonucleic acid (RNA), while the term desoxyribonucleic acid (DNA) is used for other variety with desoxypentose.

In the normal epidermis the RNA content is highest in the stratum basale, gradually decreasing in the upper layers. In active hair follicles, too, the lowest part, especially the matrix, is stained most intensely by the methods for the histochemical demonstration of RNA (Brachet 1940, 1942, Montagna 1956, Steigleder 1957). The Feulgen technique for DNA reveals the nuclei of the cells in these locations (Montagna 1956). This also applies to the nuclei of leukocytes, the cytoplasm of which shows an intense reaction for RNA, especially during mitosis (Wachstein 1955).

In guinea-pig skin after injury with solid carbon dioxide, an increase in the RNA of the epidermis has been reported to start on the fourth day (Firket 1950, 1951 b), simultaneously with the maximal proliferation of the cells in the basal layer. In healing burns in the skin of the rat an intensified staining reaction for cytoplasmic RNA has been observed as early as the third day after the injury in the mitotic cells of the basal and lower spinous layers (Washburn 1954). Likewise, regenerating fibroblasts (Bunting and White 1950) show an augmentation of RNA, whereas the DNA concentration of the nuclei does not appear to vary appreciably (Washburn 1954). Dumont (1959), however, has observed an accumulation of extracellular DNA in the vicinity of skin

wounds in the rat from the twelfth to the thirtieth postoperative hour.

Indirect evidence implicating nucleic acids in protein synthesis has been produced by histochemical (Brachet 1940, 1942, 1947, Kurnick 1955 a) and histophysical (Schultz and Caspersson 1939, Caspersson 1947) methods. Direct evidence is afforded by the abolition of protein synthesis by treatment with ribonuclease (Gale and Folkes 1953).

2. HISTOCHEMICAL METHODS

The difference between the two alternative sugar components of the nucleic acids has been used for the histochemical demonstration of DNA. The Feulgen reaction (Feuglen and Rossenbeck 1924) is thought to depend on the uncovering of the potential aldehyde group of desoxyribose by hydrolysis of purine-desoxy bonds with hydrochloric acid (Kurnick 1955 b). The aldehyde gives a purple colour when treated with fuchsin-sulphurous acid (Schiff's reagent). Despite some criticism (Danielli 1947, Stedman and Stedman 1947, Ely and Ross 1949, Lhotka and Davenport 1951), many authorities (Kurnick 1955 b, Pearse 1960) consider that for the qualitative or semiquantitative localization of DNA the Feulgen reaction is still the method of choice.

Since the nucleic acids are basophilic on account of the free phosphate groups, any basic dye may be employed for their demonstration, which depends on the formation of salt linkages ((Stearn and Stearn 1929). The staining of nucleic acids with methyl green and pyronin has been known since the beginning of this century (Pappenheim 1899, Unna 1902). It has lately been shown that chloroform-extracted methyl green is a selective stain for DNA, and chloroform-washed pyronin Y preparations for RNA, when a mixture of these dyes is used (Kurnick 1950, 1952, 1955 b).

However, with a few exceptions, basic dyes will not readily distinguish between the nucleic acids and other acid substances. The value of some of these methods may be enhanced by comparing the original preparation with one stained identically after appropriate extraction procedures. Among these the specificity of enzyme extractions, either with ribonuclease (Brachet 1940, 1942) or with desoxyribonuclease, has nowadays usually been

recognized (Kurnick 1955 b, Pearse 1960). The removal of variable amounts of RNA by electrolytes in the solvent (Stowell and Zorzoli 1947) has been avoided by using ribonuclease in glass-distilled water.

The following techniques were used in this study for the demonstration of nucleic acids:

The halves of skin flaps (see Experiments) were fixed for 24 hours at room temperature in neutral buffered 10 per cent formalin. Dehydration and clearing were performed in the usual manner. The specimens were embedded in paraffin at a relatively low temperature (+ 52° to + 54°C) in order to avoid depolymerization of the nucleic acids (Kurnick 1955 b). The sections, 10 μ in thickness, were treated by four different methods:

1) Feulgen technique (Pearse 1960). The time of hydrolysis was eight minutes. Schiff's reagent was prepared according to the modification of de Tomasi (1936).
2) the methyl green-pyronin method (Kurnick 1955 c). Edward Gurr's (chloroform-extracted) pyronin Y was used.
3) ribonuclease extraction (Pearse 1960), and subsequent methyl green-pyronin staining. The commercial salt and protease-free preparation Ribonuclease ® (Sigma Chemicals), derived from bovine pancreas and crystallized five times, was used as the enzyme source. The sections were incubated in a freshly prepared solution of this (0.5 mg/ml in glass-distilled water) for one hour at + 37°C. After staining, they were compared with control sections incubated in the solvent alone.
4) desoxyribonuclease extraction (Jackson and Dessau 1955), followed by Feulgen staining. The incubation medium was prepared by dissolving streptococcal enzyme (Varidase ®, Lederle) in 0.025 M-veronal buffer (pH 7.5) at a concentration of 1000 units per ml. The buffer contained 0.003 M-$MgSO_4$ (Pearse 1960). The mixture was dropped on the slides so as to cover the sections, and replaced every 15 minutes during the incubation time of three hours. Control sections were incubated without the enzyme. After washing, dehydrating, and covering with celloidin the treated and control sections were stained for comparison.

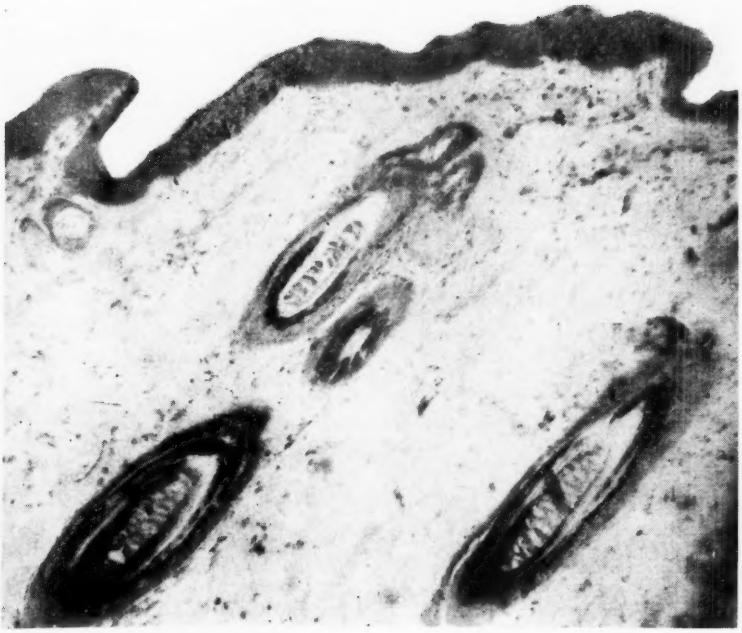

Fig. 25. — Nucleic acids in guinea-pig skin. (Methyl green-pyronin staining, × 100)

3. RESULTS

The areas staining red by the methyl green-pyronin method, and remaining without that colour after ribonuclease extraction, were considered to contain RNA. The sites which stained green by the methyl green-pyronin technique, and reddish purple by the Feulgen method were regarded to contain DNA, provided that the last-mentioned colour was abolished by desoxyribonuclease.

In the epidermis of the uninjured skin the cytoplasm of the cells in the stratum Malpighii showed a positive reaction for RNA (2 plus in the str. basale, 1 plus in the upper layers), while the stratum corneum was devoid of it. In active hair follicles the matrix stained intensely (3 plus), and the upper bulb moderately (2 plus) for RNA (Fig. 25). Some (1 plus) pyroninophilia was observed in the proximal part of the inner root sheath, whereas that part of the outer one only occasionally showed RNA. The distal part of the outer root sheath stained like the stratum Malpighii

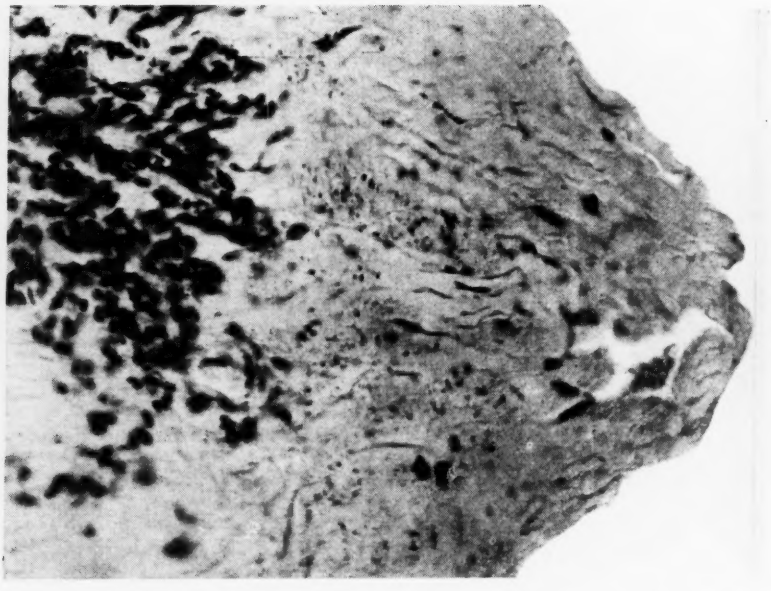

Fig. 26. — Small extracellular Feuglen-positive particles in the central zone of a 32-hour vital wound. The nuclei of the cells in the peripheral zone are visible on the left. (Feulgen, × 400)

(1 plus to 2 plus). The peripheral cells of the sebaceous glands reacted intensely (3 plus) for RNA, and the cytoplasm of the dermal connective tissue cells also took up the stain (1 plus).

In the stratum basale the nuclei were more (2 plus) Feulgen-positive than in the upper layers of the stratum Malpighii (1 plus). The nuclei in the inner root sheath lose their stainability above the middle of the hair follicle although those of the outer sheath were still Feulgen-positive (2 plus) above this level. The peripheral cells of the sebaceous glands and the dermal fibroblasts had moderately (2 plus) reactive nuclei.

No distinct changes were observed until eight hours had elapsed since the vital injury. At that time polymorphonuclear leukocytes with Feulgen-reactive (2 plus) nuclei and weakly (1 plus) pyroninophilic cytoplasm appeared in the peripheral wound zone. Some diffuse pyroninophilia was seen in the inner zone.

In 16-hour vital wounds the polymorphonuclear leukocytes were largely replaced by mononuclear phagocytes. These cells, together with the dermal fibroblasts, established a definite periph-

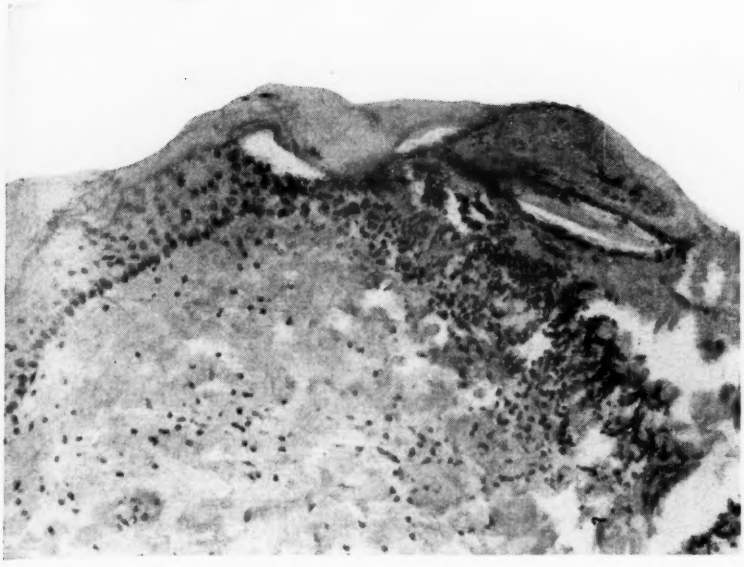

Fig. 27. — Feulgen-positive nuclei in a 32-hour vital wound. (Feulgen, × 100)

eral zone, in which the nuclei showed a 2-plus Feulgen reaction, and the cytoplasm coloured 1 plus with pyronin. In the central zone minute Feulgen-positive (1 plus) extracellular particles appeared (Fig. 26), and the diffuse pyroninophilia was unaltered. The connective tissue cells of that area began to lose the reactions demonstrating DNA and RNA, whereas the epidermal cells retained their stainability.

In 32-hour vital wounds fibroblasts and mononuclear phagocytes still dominated in the peripheral zone with their 2-plus Feulgen-positive nuclei. The pyroninophilia of these cells increased up to 2 plus. The cells of the stratum Malpighii in the peripheral zone also showed an intensified (3 plus) reaction for RNA. In the central zone the connective tissue cells exhibited almost no stainability for DNA or for RNA, and even the diffuse pyroninophilia seen there disappeared. The cells of the stratum basale and of the active hair follicles in the central zone retained unchanged both their Feulgen-reactivity (Fig. 27) and their ability to stain with the methyl green-pyronin method (Fig. 28).

Sixty-four hours after the vital injury the Feulgen reactivity continued to be 2 plus in the nuclei of the peripheral zone. The

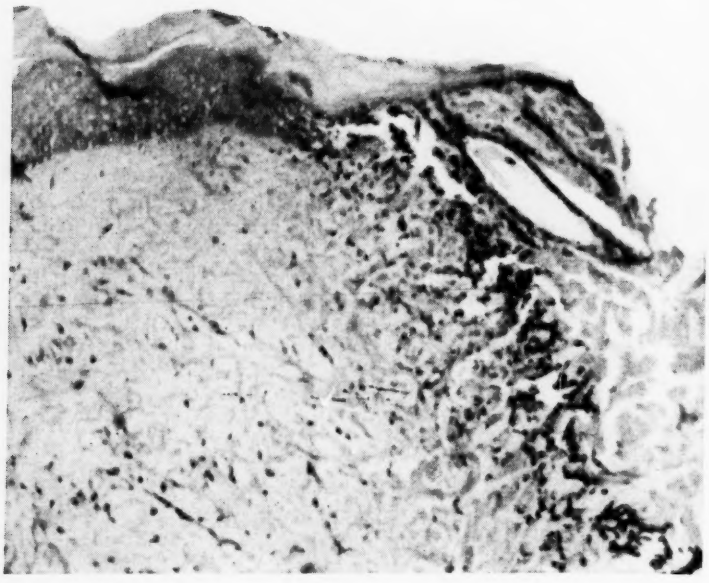

Fig. 28. — Methyl green-pyronin staining of the same wound as shown in figure 27. (× 100)

only change was that the nuclei were still closer together. The cytoplasm of these cells showed an intense (3 plus) reaction for RNA both in the stratum Malpighii and in the dermis. In the central zone the stainable RNA and DNA had disappeared from the connective tissue cells, and in the stratum Malpighii and in the active hair follicles both the Feulgen reaction (1 plus) and the pyroninophilia had likewise become fainter (1 plus).

The reactions for DNA and RNA in 128-hour vital wounds were similar to those observed 64 hours earlier. Here, too, the depth of the peripheral zone varied between 100 μ and 300 μ, that of the central zone between 200 μ and 500 μ.

The vital changes were clearly demonstrable up to five days after death. No such phenomena were seen in the wounds made post mortem.

4. DISCUSSION

The fact that pyronin stains depolymerized DNA, from which it is not displaced by methyl green (Harris and Harris 1950, Kurnick 1955 b), might account for the diffuse pyroninophilia seen in

the inner zone between eight and thirty-two hours after wounding. The death of the cells of this area was also evidenced by the appearance of Feulgen-positive extracellular particles between sixteen and thirty-two hours after the injury. These fragments may represent nuclear debris, *e.g.* of the polymorphonuclear leukocytes, which become replaced by mononuclear cells at that time. The loss of Feulgen-reactivity by the nuclei and of RNA-stainability by the cytoplasm of the connective tissue cells, beginning in the central zone 16 hours after the vital injury, and completed 64 hours post-operatively, may be compared with the results of biochemical studies by Stowell, Berenbom and Chang (1954). According to these workers, RNA decreases by 88 per cent during 48 hours in advancing necrosis of the liver, DNA disappearing more slowly. On the other hand, the epidermal cells and those of the external rooth sheath, which are homologous with them, seem to be more tenacious than the dermal cells in preserving their stainability for DNA and RNA.

The increase in the staining for RNA of the epidermal and connective tissue cells of the peripheral zone after 32 hours may be connected with the participation of RNA in the synthesis of cytoplasmic protein, without which proliferation can not occur. Opinions differ, however, as to whether a net increase in RNA is a prerequisite for protein synthesis (Barka, Törö and Pósalaky 1953, Wilson *et al.* 1953, Yokoyama *et al.* 1953, Pardee 1954) or not (Jeener 1952). As a compromise, Borsook (1956) and Zamecnik *et al.* (1958) have considered that only part of the cytoplasmic RNA is concerned in the incorporation of amino acids into new protein molecules.

The unchanged Feulgen reactivity of the nuclei in the peripheral zone is quite compatible with the well known observations (*e.g.* by Mirsky and Ris 1949) that the DNA content is constant in the nuclei of most organs of a single species, and that it is unaffected by many changes which greatly alter the amounts of RNA.

In the central zone, except for some extracellular remnants of nucleic acids visible after eight hours, the histochemical tests for these substances revealed a decrease in the stainability of the connective tissue cells from 16 hours, and in that of the epidermal cells from 64 hours after vital injury (negative vital reaction). Further, in the peripheral zone these methods showed an

increase in the cytoplasmic RNA from 32 hours, intensifying up to 64 hours, and an accumulation of Feulgen-positive nuclei from eight hours after wounding (positive vital reaction). Seeing that no such phenomena were observed in the wounds made post mortem, and since the vital changes were recognizable up to five days after death, the histochemical demonstration of nucleic acids might be used as a supplementary method in the distinction between vital and post-mortem skin wounds.

5. SUMMARY

In the uninjured skin the stratum Malpighii, some cutaneous appendages, and connective tissue cells stained for RNA and DNA. In the injured skin the peripheral wound zone consisting of the stratum Malpighii in the epidermis, and of connective tissue cells in the dermis, showed an increased stainability of the cytoplasmic RNA beginning in 32-hour vital wounds, and intensifying up to 64 hours. This, and in addition the accumulation of Feulgen-positive nuclei after eight hours, was called the positive vital reaction. In the central zone the stainability for both RNA and DNA of the connective tissue cells decreased from 16 hours, and that of epidermal cells from 64 hours after the vital injury (negative vital reaction). These vital changes were detectable for five days after death and no such zones were seen in the wounds made post mortem. The histochemical demonstration of nucleic acids is thus of supplementary value in the medicolegal distinction between vital and post-mortem skin wounds.

XI. STUDIES ON ACID MUCOPOLYSACCHARIDES

1. EARLIER INVESTIGATIONS

Ground substance is the amorphous mass separating the cells, fibres and vessels of connective tissue, and containing protein, mucoprotein and mucopolysaccharides. The latter, which are the most typical components of the ground substance, have hexosamine as one constituent, and the prefix »muco» indicates their mucinlike nature (Dorfman 1953, Meyer 1954, Hale 1957). Mucopolysaccharides may be neutral, containing only hexose and hexosamine, or acid, consisting of hexosamine, a uronic acid, and, in some cases, an ester sulphate. Meyer (1959) distinguished two categories of acid mucopolysaccharides: (1) non-sulphated, *e.g.* hyaluronic acid, and (2) sulphated, including chondroitin sulphates A, B and C. His classification depends partially on the differential resistance to hyaluronidases which are hydrolyzing and depolymerizing enzymes, usually either of bacterial or testicular origin, used in the study of mucopolysaccharides. Bacterial hyaluronidase removes hyaluronic acid but not the sulphated acid mucopolysaccharides. Among the latter, chondroitin sulphates A and C are affected by the testicular enzyme, whereas chondroitin sulphate B is resistant even to it.

According to biochemical investigations (Meyer and Chaffee 1941, Pearce and Watson 1949, Meyer 1959), the bulk of the skin mucopolysaccharides is composed of chondroitin sulphate B, and of hyaluronic acid. Histochemically, in the normal skin the stratum Malpighii of the epidermis and some epithelial and stromal elements of the active hair follicles stain for acid mucopolysaccharides (Montagna 1956). This material is associated with periods of intense proliferation of hair epithelium, and it is most likely supplied

via the stromal matrix (Sylvén 1950). Small amounts of acid mucopolysaccharides are also stainable within the dermis, being most conspicuous in the papillary layer and around hair follicles (Bunting 1950, Braun-Falco 1958).

In healing skin wounds in rats an increase in acid mucopolysaccharides has been histochemically observed to start on the first postoperative day, reaching a peak on the fifth (Sylvén 1941, Dunphy and Udupa 1955) or sixth to the ninth days (Balazs and Holmgren 1950). In mice, after cutaneous excisions, fibroblasts are reported to show increased numbers of polysaccharide-containing granules for about nine days, after which they tend to return to normal (Gersh and Catchpole 1949). Bunting and White (1950) have studied skin wounds in the guinea-pig from the third to the 16th postoperative day. According to them, acid mucopolysaccharides, which are »especially conspicuous in the early samples», are observed between the fibroblasts and the capillaries, appearing to coat the connective tissue fibres. The granulation tissue of the dog, forming within wire mesh cylinders, shows a very faint stainability for acid mucopolysaccharides in the first week, the colour becoming distinct by the end of two weeks, and reaching its maximum intensity at three weeks (Schilling, Joel and Shurley 1959). In a recent study, an increase in acid mucopolysaccharides has been encountered »hand-in-hand with proliferation of fibroblasts» since the third postoperative day in incisions in rabbit skin (Movat, More and Wolochow 1960). According to several authors (Penney and Balfour 1949, Bunting and White 1950, Campani and Reggiani 1950) metachromatic material indicating the presence of acid mucopolysaccharides, is removable by testicular hyaluronidase in healing wounds.

Several functions have been ascribed to mucopolysaccharides in wound healing. Sulphated polysaccharides could act as cation exchangers (Meyer and Rapport 1951) or by supplying sulphurous constituents for keratin synthesis (Sylvén 1950, Montagna 1956). The non-sulphated hyaluronic acid may play some role, *e.g.* in water-binding (Meyer 1954, Edwards and Dunphy 1958). However, the most important function of the mucopolysaccharides in healing is as precipitating agents necessary for the formation of collagen (Highberger, Gross and Schmitt 1951, Dunphy and Udupa 1955, Jackson 1958, Gross 1959).

2. HISTOCHEMICAL METHODS

Metachromasia is the phenomenon by which certain dyes in solution react with a substance to produce a colour that is different from that of the original dye solution (Ehrlich 1877). When diluted aqueous toluidine blue is used, the term refers to a change in colour from blue, through violet, to red, in tissues containing such substances as acid mucopolysaccharides. According to modern views (Sylvén 1954, Kelly 1956, Pearse 1960), this reaction signifies only the presence of free electronegative surface charges of a certain minimum density. Especially the alcohol-stable or »true» metachromasia is claimed to be largely due to ester sulphates (Sylvén 1941, 1950, Pearse 1960). In addition, the non-sulphated hyaluronic acid is also believed to react like the chondroitin sulphates in this respect, at least in high concentrations (Meyer 1947, Wislocki, Bunting and Dempsey 1947, Bunting 1950, Sylvén and Malmgren 1952). The information afforded by metachromasia regarding the presence of acid mucopolysaccharides may be supplemented by using hyaluronidase extractions with subsequent staining for comparison.

Alcian blue 8 G S, a phthalocyanin dye, combines with acid groups either by a salt linkage (Pearse 1960) or by an amide bond (Spicer 1960). It seems to be the most specific stain in use for the demonstration of acid mucopolysaccharides (Steedman 1950, Braun-Falco 1957 d, Pearse 1960). By contrast, the specifity of the methods used by Hale (1946) and by Rinehart and Abul-Haj (1951), based on the affinity of these substances for colloidal iron, is doubtful (Braden 1955, Pearse 1960), and the methods depending on the oxidation of 1,2-glycol groups, *e.g.* the PAS technique of McManus (1946, 1948), do not demonstrate acid mucopolysaccharides (Glegg, Clermont and Leblond 1952, Weissmann and Meyer 1954, Braun-Falco 1957 d).

Comparative studies have revealed that most of the usual fixatives, including formalin, are entirely satisfactory for the staining methods demonstrating acid mucopolysaccharides (Kramer and Windrum 1955, Schubert and Hamerman 1956, Kelly 1956).

The following procedure was used in this study for the demonstration of these substances.

The specimens were fixed at room temperature in neutral buffered 10 per cent formalin for 24 hours. Dehydration, clearing, and embedding in paraffin were performed in the usual manner. The 10 μ sections were treated by the following methods:

1) Lison's Alcian blue technique (Lison 1954).

2) toluidine blue for metachromasia, modified according to Bunting and White (1950). The sections were brought to water, stained in 0.05 per cent aqueous solution of toluidine blue for 16 hours, rinsed for three seconds in 96 per cent alcohol, dehydrated three times, for three seconds each, in absolute alcohol, placed directly in xylene, and mounted.

3) hyaluronidase extractions (Pearse 1960) and subsequent toluidine blue staining. The testicular hyaluronidase preparation Hyalase® (Benger) was dissolved in 0.85 per cent sodium chloride solution at a concentration of 1500 IU per 3 ml. As a bacterial hyaluronidase, Hyason® (Organon) was used. The contents of each ampoule of this product (150 T.R.U.) were dissolved in 1.5 ml of 0.85 per cent sodium chloride solution. After three hours' incubation at + 37°C in a medium containing one or other of these hyaluronidases, the washed sections were stained for 20 minutes in 0.5 per cent aqueous toluidine blue and compared with identically stained control sections which had been incubated for three hours at + 37°C in saline only.

3. RESULTS

The sites staining bluish-green with Alcian blue and pink or reddish with toluidine blue were considered to contain acid mucopolysaccharides.

In the uninjured skin the stratum Malpighii and the outer rooth sheath stained 1 plus, the dermal papilla and the connective tissue sheath, especially around the middle third of the follicle, 2 plus for acid mucopolysaccharides. Scattered foci in the stratum papillare of the dermis showed a faint (1 plus or less) colour indicating the presence of these substances. The metachromasia at these sites resisted both the hyaluronidases used.

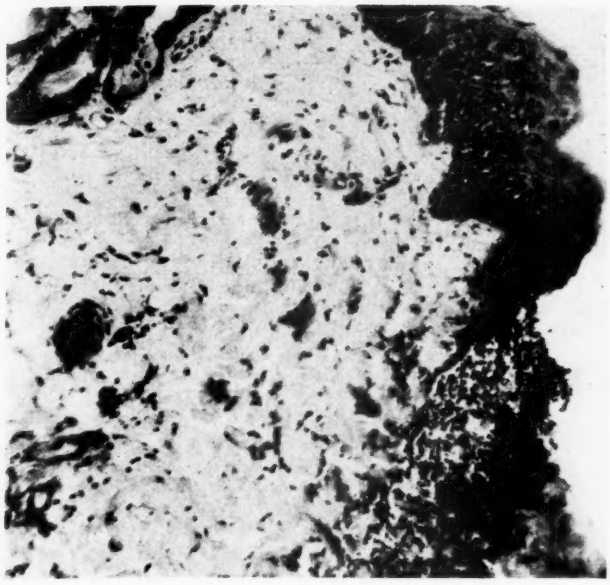

Fig. 29. — Acid mucopolysaccharides in a 32-hour vital wound. (Toluidine blue, × 100)

In the wounded skin there were no recognizable changes in acid mucopolysaccharides until 32 hours after the vital injury (Fig. 29). At that time both the stratum Malpighii of the epidermis and the dermis showed an intensified (2 plus) colour in the peripheral wound zone with both of the staining methods. The depth of this area was of the order of 100 μ to 300 μ. In the central zone, varying between 200 μ and 500 μ, loss of acid mucopolysaccharides was beginning (1 plus or less in the stratum Malpighii, no reaction in the dermis).

Sixty-four hours after the vital injury no further increase in these substances was demonstrable in the peripheral zone. At the bottom of the wounds acid mucopolysaccharides lay between the mononuclear phagocytes and fibroblasts and, in addition, in the cytoplasm of the latter cells (Fig. 30). In the central zone there were no stainable acid mucopolysaccharides. The picture in the 128-hour vital wounds was on the whole similar to that seen 64 hours earlier. The granulation tissue showed a 2 plus staining for these substances between the capillaries and the numerous fibroblasts.

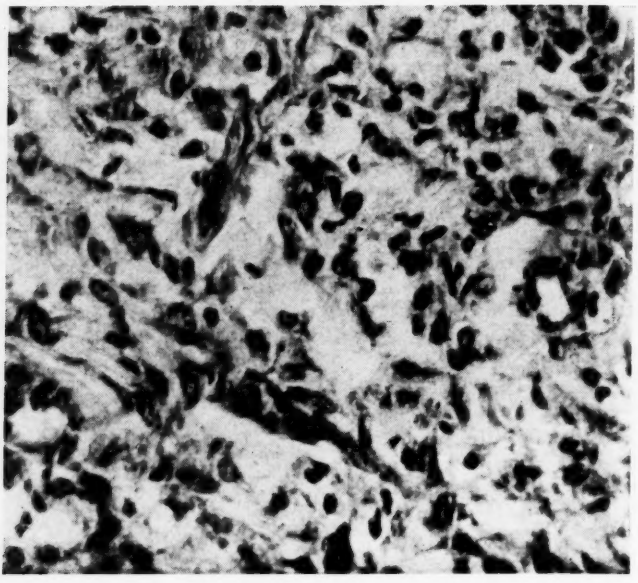

Fig. 30. — Acid mucopolysaccharides lying in the cytoplasm of fibroblasts and between these and other mononuclear cells at the bottom of a 64-hour vital wound. (Toluidine blue, × 400)

The increased metachromasia of vital wounds was removed by the testicular but not by the bacterial hyaluronidase.

The location and intensity of the stains indicating acid mucopolysaccharides remained roughly unchanged up to five days after death. No false increase in staining could be demonstrated in the wounds made post mortem.

4. DISCUSSION

The metachromasia of the uninjured skin resists both types of hyaluronidases but the increased metachromatic staining in vital wounds is removable by the testicular enzyme. The latter phenomenon might indicate that the material accumulating in wound healing is either chondroitin sulphate A or C, or hyaluronic acid (Pearse 1953, 1960, Raekallio 1958). However, the testicular enzyme does not diminish the metachromasia of the »normal» skin as it should on account of the hyaluronic acid content. The

discrepancy between the biochemical and histochemical results is probably an indication that the removable material in tissue sections is not so readily accessible to enzyme digestion as in biochemical experiments. Thus, it does not seem justifiable to distinguish between the various acid mucopolysaccharides by the histochemical methods.

Mucopolysaccharides are produced either by mast cells (Sylvén 1941, Asboe-Hansen 1954) or by fibroblasts (Stearns 1940, Gersh and Catchpole 1949, Bunting and White 1950). The results of certain recent studies argue against the former theory. Fisher and Hellstrom (1961) have shown, that even effective depletion of tissue mast cells in rats failed to influence the appearance of acid mucopolysaccharides in the healing wounds. The latter theory has gained support from experiments made with radiosulphur (Layton 1950, Glücksmann 1959). In the present study, too, large amounts of acid mucopolysaccharides have been observed in the fibroblasts or in their immediate vicinity.

Among the many possible functions of the mucopolysaccharides in wound healing, their role in the formation of collagen is of prime importance. One might thus say that »ground substance» is a mistranslation of the German word »Grundsubstanz». Perhaps the term fundamental substance would better describe the versatile and essential activities in which the mucopolysaccharide components of the so-called ground substance are involved.

In the peripheral wound zone the histochemical methods for acid mucopolysaccharides showed an increase in stainability visible from 32 hours after the vital injury. In conformity with the description of the other results reported in this work, this may be called a positive vital reaction. In the central zone there was a loss of staining beginning 32 hours after vital wounding, and no acid mucopolysaccharides were demonstrable in that area after 64 hours (negative vital reaction). The vital changes were detectable up to five days after death and no such zones were observed in the wounds made post mortem. However, the changes revealed by the histochemical demonstration of acid mucopolysaccharides seem to require for their manifestation the elapse of a relatively long time between the injury and the death of the victim, if these methods are to be used for the medicolegal distinction between vital and post-mortem wounds.

5. SUMMARY

The stratum Malpighii and the growing hair follicles were the principal sites of acid mucopolysaccharides in the uninjured skin. In the wounded skin the peripheral zone exhibited an intensified stainability for these substances from 32 hours after the vital injury (positive vital reaction). In the central zone the loss of staining began at the same point of time, and 64 hours after vital wounding no acid mucopolysaccharides were demonstrable in that area (negative vital reaction). The vital changes were recognizable for five days after death, and no such phenomena occurred in the wounds made post mortem. According to these experiments, the histochemical demonstration of acid mucopolysaccharides did not seem to be very useful for the practical distinction of vital from post-mortem wounds.

XII. GENERAL DISCUSSION AND CONCLUSIONS

According to the results reported, in vital skin wounds of the guinea-pig it was possible to distinguish a central zone and a peripheral zone. In the central zone a progressive loss of stainability occurred. By demonstrating enzyme activities, the decrease could be made visible as little as two to four hours after the injury. In 8-hour wounds the conventional histological Weigert—van Gieson technique showed the first signs of necrosis, this being more advanced in the connective tissue cells after 16 hours. It thus seemed apparent that the methods of enzyme histochemistry were sensitive enough to detect an early phase of regression in the central wound zone. The diminished vitality of its cells might partly be due to the actual damage by wounding, partly to bacterial infection. In addition, the reduction in blood supply caused by local destruction of blood vessels probably plays a role in the decline of cell activity, until it becomes compensated by the growth of new capillaries in the damaged area.

In the peripheral zone an increase in the activity of hydrolases could be noted after two to eight hours. The accumulation of invading leukocytes was not apparent until 8 to 16 hours after wounding. Hence, the main source of the increased enzyme activity must be a local tissue element. Among the local cells, the fibroblasts play a leading role, showing the strongest reactions. Montagna (1956), indeed, calls the fibroblast a master cell of the connective tissue, since it is able to undergo countless modifications in response to environmental demands. In this accommodation, enzymatic adaptation, *i.e.* the ability of individual cells to change their enzymatic makeup to fit the environment (Knox, Auerbach and Lin 1956, Potter and Auerbach 1959) is of prime importance.

The same trauma that causes regression in the central zone thus seems to stimulate the cells of the peripheral zone. In addition to local cells, the invading polymorphonuclear and mononuclear cells appeared to participate, in a later phase of healing, in the intensification of the staining reactions, especially those for the activity of oxidative enzymes, for acid mucopolysaccharides and RNA.

It is impossible, or at least difficult, solely by histochemical methods to settle the question of whether the enzymatic adaptation in wound healing involves an actual increase in the amount of the enzymes present, in addition to the intensification of their activity. As a matter of fact, the amount of a single enzyme is seldom the only limiting factor in a histochemical reaction. At least in microorganisms, however, enzymatic adaptation involves synthesis *de novo* of a specific protein from amino acids (Rotman and Spiegelman 1954, Hognes, Cohen and Monod 1955). Regarding the increased stainability of acid mucopolysaccharides and RNA, it is also difficult to decide whether it represents a real increase in the amount of the substance or phanerosis only. By means of proteolytic enzymes, for instance, the healing process might set free, and so make accessible to the dyes, some active groups of acid mucopolysaccharides and nucleic acids which had previously been unstainable owing to combination with protein. However, the results of certain biophysical (Caspersson 1947) and biochemical (Dunphy and Udupa 1955) investigations support the idea that an actual increase in the amount of RNA and acid mucopolysaccharides is involved in healing.

The adaptively increased enzyme activities may have several functions. Hydrolases might cause hydrolytic breakdown of certain cell constitutients. In this connection it is interesting to note that aminopeptidase showed the earliest increase in activity. Either leukotaxin (a polypeptide, cf. Menkin 1936, 1955) or histamin (Lewis and Grant 1924), both of which are possible decomposition products of proteins, is thought to give rise to local hyperaemia, increase in capillary permeability, and migration of leukocytes. In any case, intense enzyme activities were seen previously to a distinctly visible leukocytic zone. According to Needham (1952), the main function of alkaline phosphatase *in vivo* is clearly not to deposit inorganic phosphate but to liberate energy by rupture of energy-rich bonds. Weiss and Fawcett (1956) have ascribed the

enhanced phagocytic power to an increase in acid phosphatase activity.

In addition to their hydrolytic action in the defensive system of the peripheral wound zone, the hydrolases very probably participate in synthetic repair processes. Alkaline phosphatase, especially, has been related to the synthesis of fibrous proteins, for instance in the formation of collagen by fibroblasts.

Cytochrome oxidase and succinate dehydrogenase participate in the respiratory processes of cells, which may explain their intense activity in the vivid peripheral zone. In the beginning of the healing process, however, the tissue reaction is acid and therefore anaerobic glycolysis replaces aerobic oxidation (Needham 1952). This is probably the reason for the relatively late increase in the activity of the oxidative enzymes

Aerobic oxidation is necessary for the occurrence of mitoses in normal tissues (Bullough and Johnson 1951). This may explain the intense activity of the oxidative enzymes observed in the proliferating stratum Malpighii. The increased stainability of RNA in the same layer might be connected with the participation of RNA in the synthesis of cytoplasmic protein, the latter being essential for proliferation. Mucopolysaccharides, which are probably produced by the fibroblasts, are considered to be precipitating agents for collagen fibrils (Jackson 1958).

According to the physical and histological investigations referred to in the introduction, the length of the »lag» phase, during which healing did not seemingly progress ranged from four to seven days after wounding. Some biochemical and histochemical observations reduced this period to about one day. The methods of enzyme histochemistry used in this study seem now to shorten the »lag» period to some hours, if it is assumed that hydrolases also have a synthetic action, *i.e.* that these enzymes play a role in a true repair process. Even if this suggestion is not acceptable, however, the reality of the metabolic inertia or even more the »regression» ascribed to the early days following injury, may be questioned. By contrast, in these experiments with guinea-pigs an increase in the activity of oxidative enzymes was seen in the peripheral zone of vital wounds as little as eight hours after the injury. This indicates invigorated cell respiration. In the central area, it is true, actual regression took place during the experimental period.

Thus, the concept »lag phase» is a relative one. In the central zone of the wound it unquestionably exists. In the peripheral zone, on the other hand, its length depends on the phenomenon studied, *e.g.* the appearance of hydrolases or the increase in the mitotic count.

In the central zone of vital wounds, a decrease in stainability did occur. The onset of this regression varied from two (for aminopeptidase activity) to 32 hours (for acid mucopolysaccharides) after the injury, being four hours for most of the enzymes studied. Since no such decrease was observed in the wounds made post mortem, these manifestations of regression were called negative vital reactions. In the peripheral zone, an intensification of the staining reaction was shown to begin from two (aminopeptidase activity) to 32 hours (RNA and acid mucopolysaccharides) after the vital injury. These increases in stainability were called positive vital reactions, because no such phenomena were observed in wounds made after death.

According to the experiments performed, the histochemical demonstration of the changes in the activity of aminopeptidase and acid phosphatase revealed the earliest vital reactions. As the vital phenomena were recognizable for five days after death, *i.e.* for the period during which most medicolegal autopsies are performed, it seems possible that the results obtained might be applied in practice to the distinction between vital and post-mortem skin wounds. Before this, however, because of possible specific differences, extensive studies are necessary on medicolegal autopsy material from which, as far as possible, exact data are available concerning the moment of the injury and of the victim's death.

In the light of the reported results and of the foregoing discussion the following conclusions seem justifiable:

1. In the central wound zone of 200 μ to 500 μ depth, vital trauma caused a gradual loss of stainability. This possibly represented a decreasing activity of cells, which was generally demonstrable as a true »lag» period from the fourth hour on by the methods of enzyme histochemistry, or from the 16th and 32nd hours on by means of staining reactions for nucleic acids and acid mucopolysaccharides, respectively.

2. In the 100 μ to 300 μ deep peripheral zone the same injury evoked an increase in stainability by the methods for demonstrating the activities of some hydrolytic and oxidative enzymes, from the second to the eighth hour on after wounding. The metabolically inert »lag» phase in the outer zone was, thus, reduced to a few hours.

3. The greater part of the intensified activity of the hydrolases studied seemed to arise locally. The initial increase was probably due to an enzymatic adaptation, principally on the part of the fibroblasts. From eight to 16 hours after the vital injury, polymorphonuclear leukocytes and mononuclear invading cells began to appear in the peripheral zone. These obviously contributed to the intensification of the enzymatic reactions from the eighth hour on. Both the local and the migrating cells probably caused the increase in demonstrable RNA and acid mucopolysaccharides found in 32-hour vital wounds.

4. Neither the increased stainabilities in the peripheral zone nor the regression in the central zone were observed in wounds made after death. Therefore, the former phenomena can be called positive vital reactions, and the decreased stainabilities negative vital reactions.

5. Both types of vital reactions were recognizable for five days post mortem.

6. The animal experiments performed showed that the earliest histochemically demonstrable vital reactions were the changes in aminopeptidase activity after two hours and in acid phosphatase activity after four hours. These two histochemical methods might thus be worth trying in the medicolegal distinction between vital and post-mortem human skin wounds. The other methods described could give supplementary evidence of the vital origin of a wound. and make up a biological time-table which might possibly be useful, *e. g.*, in the reconstruction of homicide cases.

XIII. SUMMARY

Many of the methods previously used for distinguishing vital from post-mortem wounds have been based on phenomena which appear relatively late or, unfortunately, also in the wounds made after death. In addition, certain physical and histological investigations have led to the doctrine of the metabolically inert »lag» phase that was supposed to last for several days after the injury.

It seemed possible, that the earliest changes had escaped observation because the previous methods had not been sensitive enough. The newer histochemical methods seemed to offer a possibility to detect some of the very first reactions, and provide useful information for the medicolegal distinction between vital and post-mortem skin wounds. Therefore, the following points were investigated:

1) the appearance of certain histochemical changes in vital skin wounds
2) the exclusion of similar phenomena possibly appearing in wounds made post mortem,
3) the demonstrability of such changes within reasonable periods after death.

An experimental study was made on guinea-pigs by excising circles of the dorsal skin. Nine animals were killed 1/2, 1, 2, 4, 8, 16, 32, 64 or 128 hr. after wounding, and skin flaps each containing one wound were removed immediately. Similar excisions were then made on the other side of the same animals at equal intervals after death. The tenth guinea-pig was killed 2 days after wounding, and the skin flaps were removed from this animal 1, 2, 3, 4 or 5 days after death. Four replicate series of experiments

were made, and the material thus consisted of 40 animals. To every wound 14 histological or histochemical techniques were applied.

The chief results obtained were:

1. In the central wound zone, 200 to 500 μ in depth there was a gradual loss of stainability. This was demonstrable from the following times on: aminopeptidase avtivity 2 hr., acid and alkaline phosphatase, cytochrome oxidase and succinate dehydrogenase activity 4 hr., nucleic acids 16 hr., and acid mucopolysaccharides 32 hr. after the vital injury. Histologically, signs of the onset of necrosis were visible after 8 to 16 hr.

2. In the 100 μ to 300 μ deep peripheral zone a vital increase in the enzyme activities appeared, mainly in fibroblasts, from the following times on: aminopeptidase 2 hr., acid phosphatase 4 hr., alkaline phosphatase, cytochrome oxidase, and succinate dehydrogenase 8 hr. after wounding. A vital intensification of stainability for ribonucleic acid and acid mucopolysaccharides was seen 32 hr. after the injury. Histologically, after 8 to 16 hr., polymorphonuclear and mononuclear cells began to migrate into the outer zone. Mitoses were observed, chiefly in the basal layers of the epidermis, in 64 hr.-vital wounds.

3. Neither the increased stainabilities in the peripheral zone nor the regression in the central zone were observed in wounds made after death. Therefore, the former phenomena can be called positive vital reactions, and the decreased stainabilities negative vital reactions.

4. Both types of vital reaction were recognizable up to five days after death.

In the light of these results it appears that:

1. The decrease in stainability observed in the central wound zone signifies a gradual loss of vitality in the cells. This regression is a true »lag» phase lasting at least 128 hr.

2. The initial increase in enzyme activities is mainly due to local factors, chiefly to enzymatic adaptation on the part of the

fibroblasts. After 8 to 16 hr. migrating cells participate in the intensification of stainability, together with the local ones, including the living epidermal cells.

3. The so-called lag period in the peripheral zone appears to be reduced to a few hours in the wounds studied.

4. The histochemical methods for aminopeptidase and acid phosphatase activities, which have revealed the earliest vital reactions in the animal experiments, may prove of value for the medicolegal distinction between vital and post-mortem skin wounds also in man. The other methods used might give supplementary evidence of the vital origin of a wound, and allow the construction of a biological time-table which would be useful, *e. g.*, in the reconstruction of homicide cases.

XIV. ADDENDUM

Since it was felt that the relatively faint increase in the activities of these enzymes in early vital wounds would possibly not persist after death so well as the pronounced activity occurring in 48-hour vital wounds, a supplementary experiment seemed desirable. For this purpose, three additional guinea-pigs were treated in the way described in section III of this study. On the dorsal skin of each animal five pairs of wounds were made. The first animal was killed two hours, the second four hours, and the third eight hours after wounding. The most anterior couple of skin flaps was removed 24 hours after death, the second after 48 hours, the third three days, the fourth four days, and the fifth five days after the death of each animal. After the appropriate pre-treatment, and with the histochemical methods described previously, the activity of the following enzymes was studied: aminopeptidase in the skin flaps of the first animal, acid phosphatase in those of the second, alkaline phosphatase, cytochrome oxidase and succinate dehydrogenase in the skin flaps of the third.

The vital changes in activity persisted for five days after death in this supplementary experiments as well as in 48-hour vital wounds. This confirms result no. 4 of the summary.

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